GENETIC EFFECTS OF PROLONGED UV-B EXPOSURE IN A NAMAQUALAND DAISY - *DIMORPHOTHECA SINUATA*

By

Sununguko Wata Mpoloka

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University of Cape Town

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ABSTRACT

This thesis describes investigations into the genetic effects of long term UV-B exposure in Namaqualand daisies (*Dimorphotheca sinuata*) grown for several generations under ambient and enhanced UV-B levels. Enhanced UV-B radiation was found to have a major effect on the biochemical composition of the chloroplast accompanied by impairment of photosynthetic function, involving a down-regulation of photosynthetic genes and an up-regulation of flavonoid biosynthesis. Plants that were previously grown for four generations in the presence of enhanced UV-B radiation showed evidence of UV-B effects on various physiological processes, growth and reproduction, indicating a likelihood of these effects being heritable. Gene expression was examined in fifth generation plants grown in a greenhouse in the absence of UV-B radiation using photosynthetic gene probes (*rbc*L and *psb*A) in Northern hybridisation studies of mRNA levels to see if gene regulation had been affected by UV-B radiation. Analyses of both *rbc*L and *psb*A data showed that mRNA levels from the irradiated plants were on average, lower than those for non-irradiated plants but the difference did not attain statistical significance in both cases. The same plants were used to test for residual UV-B induced mutations using the *Dra*I assay using the *rbc*L and *18S* rRNA genes as probes. Even though the results did not indicate an immediate presence of UV-B induced mutations, greater variability was observed with the *18S* rRNA probe in plants with an enhanced UV-B history. UV-B radiation has been reported to cause several lesions in DNA including double strand breaks whose induction in turn increases the frequency of homologous recombination, hence genome rearrangements. Tests for correlation between mRNA levels for photosynthetic genes and biochemical data were conducted and a significant correlation was found with *psb*A mRNA levels and most photosynthetic pigments. Characterisation of chloroplast DNA from plant populations across a latitudinal gradient using restriction endonuclease digestion was carried out. These encompassed most of the plant's natural distribution range. Plants growing at northern latitudes (potentially higher UV-B environments) revealed striking polymorphisms when compared with plants from southern latitudes (lower UV-B environments), which may be attributed to genome rearrangements resulting from the UV-B stress. The entire *rbc*L gene from *D. sinuata* has been cloned, sequenced and further characterised.

This study is the first known attempt at developing a Southern African biological model for predicting the long-term effects of ozone depletion, and the resultant rise in UV-B radiation, on our indigenous flora.

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GENERAL ABBREVIATIONS

2βME	2 beta mecarpto-ethanol
μ	Greek letter Mu: prefix micro-
μg	microgram
μl	microlitre
bp	base pair
BSA	bovine serum albumin
ccc	covalently closed circular
CSPD	trademark for the Roche chemiluminiscent substrate
CTAB	cetylmethyl ammonium bromide
ctDNA	chloroplast DNA
Da	Daltons
DEPC	diethylpyrocarbonate
DIG	Digoxygenin
DNA	deoxynucleic acid
dNTPs	deoxynucleotide triphosphates
ds	double stranded
EDTA	ethylenediaminetetraacetic acid (disodium salt)
EtBr	ethidium bromide
g	gram
HCl	hydrochloric acid
IAPO	International Academic Programmes Office
IR	intergenic region
k	prefix kilo-
kb	kilobases
kbp	kilobase pair
kDa	kilo Dalton
l	litre
LA	Luria agar
LB	Luria broth
LHCII	Light harvesting complex II
LIR	long intergenic region

M	Molar
m	prefix milli-
min	minute
ml	millilitre
mm	millimetre
mRNA	messenger RNA
MW	molecular weight
n	prefix nano-
N.D	not determined
N/A	not applicable
nm	nanometres
nt	nucleotide(s)
NTS	non-transcribed spacer
^{o}C	degrees celcius
ORF	open reading frame
PAR	photosynthetically active radiation
PCR	polymerase chain reaction
PSI	photosystem I
PSII	photosystem II
PVPP	polyvinylpolypyrrolidone
*rbc*L	large subunit of the RUBISCO gene
RFLP	restriction fragment length polymorphism
RNA	ribooxynucleic acid
RnaseA	ribonuclease A
RUBISCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	ribulose bisphosphate
SDS	sodium dodecyl sulphate
SIR	short intergenic region
ss	single stranded
SSC	salt-sodium citrate buffer
T-DNA	transferred DNA
TE	Tris-EDTA
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA

U	unit
UB	University of Botswana
UCT	University of Cape Town
USHEPiA	University Science, Humanities and Engineering Partnerships in Africa
UV	ultraviolet
vv	volume per volume
wv	weight per volume

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

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Chapter 1

1.0 Introduction

1.1 UV radiation and ozone depletion

The UV spectrum is normally divided into three regions: the UV-C region (< 280 nm), the UV-B region (280 - 320 nm) and the UV-A region (320 - 400 nm). UV-B radiation is the most energetic component of sunlight reaching the earth's surface, and there has been an increase in solar UV-B radiation penetrating through the atmosphere over recent years due to ozone depletion (Caldwell *et al.*, 1989; Kerr and McElroy, 1993; Strid *et al.*, 1994). The increase in UV-B levels is due to an accelerating depletion of the stratospheric ozone shield and is caused by man-made air-pollutants such as chlorofluorocarbons (CFCs) (Harm, 1980; Kerr, 1988; Madronich, 1992; Stapleton, 1992; Stolarski *et al.,* 1992; Kerr and McElroy 1993). By the release of CFCs, society has the capacity to change the spectral capacity of sunlight by destroying the ozone layer in our stratosphere, which filters out much of the damaging short wavelength UV radiation.

Despite its low concentration, ozone plays a critical role in chemical and biological processes by filtering ultraviolet radiation in the 220 - 320 nm wavelength range. Stratospheric ozone absorbs much of the short-wave UV-B, so radiation of shorter wavelengths will undergo the greatest relative change when ozone is reduced. The effectiveness of ozone absorption decreases exponentially as the wavelength of radiation increases. All radiation consisting of wavelengths shorter than 280 nm is absorbed in the atmosphere. Some radiation in the UV-B region is attenuated by ozone while wavelengths longer than 320 nm are not significantly absorbed by ozone (Caldwell *et al.,* 1989).

Biological systems are vulnerable to wavelengths in the transitional range of 280 - 320 nm and are thus greatly affected by ozone losses. Lower ozone levels result in greater amounts of UV-B reaching the surface which can lead to damaging effects on humans, plants and animals (Caldwell *et al.*, 1989; Rozema *et al.*, 1997). Any

perturbation that leads to an increase in UV-B radiation demands careful consideration of the possible consequences. As a result, the UV-B region of the ultraviolet spectrum has gained in importance over other environmental factors.

The primary concern over ozone depletion is the potential impact on human health and ecosystems due to increased UV exposure. This enhanced exposure to UV-B is potentially detrimental to all living things but is particularly harmful to plants due to their obligatory requirement for sunlight for survival and their inability to move (Strid *et al.*, 1994). Increases in skin cancer, skin ageing and cataracts in human populations are expected in a higher UV-environment (Longstreth *et al.,* 1995). Lower yields of certain cash crops and undesirable effects in agriculture may result due to increased UV-B stress (Caldwell *et al.,* 1995); higher UV-B levels in the upper ocean layer may inhibit phytoplankton activities, which can have an impact on the entire marine ecosystem (Hader *et al.,* 1995). In addition to direct biological consequences, indirect effects may arise through changes in atmospheric chemistry. Increased UV-B will alter photochemical reaction rates in the lower atmosphere that are important in the production of surface layer ozone which has detrimental effects on plants (Tang and Madronich, 1995).

Public awareness of the dangers to health of exposure to ultraviolet (UV) light has increased in recent years. Although exposure to UV can have positive effects on humans (e.g. sunbathing generally induces a feeling of well being, partly due to production of endorphins, and stimulation of synthesis of vitamin D), the dangers far outweigh the benefits. The most noticeable effect of exposure is sunburn, or erytherma; more serious is the risk of skin cancer, and of damage to the eyes, and these symptoms may not surface for several years (Longstreth *et al.*, 1995). At the same time, the public has also become aware that protection from solar UV is provided by ozone in the stratosphere, but that over recent years the ozone layer has suffered depletion due to the action of man-made chemicals. The predicted result of this is that more UV-radiation from the sun will reach the earth's surface.

Reports from global ozone measurements by satellites over the period from 1979-1993 imply significant UV-B increases at high and mid-latitudes of both hemispheres, but only small changes in the tropics. There are significant latitudinal variations in

incident UV-B. For example, the amount of UV-B experienced at tropical latitudes is much greater than in temperate regions because the angle of the sun is such that there is less absorption by the atmosphere. In addition, the ozone layer itself is thinner in the equatorial regions (Green, 1983; Caldwell *et al.,* 1989; Lumsden, 1997).

1.2 Biological effects of UV radiation on plants and plant responses

Over the past few years, research has been carried out to investigate whether increases in UV-B radiation resulting from ozone depletion would have a significant impact on plants, in particular on aspects of physiology and crop yield. Experimental work has been carried out in laboratory growth chambers, where plants are grown under artificial white light, to which is added different levels of UV-B, or outdoors, using artificial UV-B to supplement the UV-B in natural sunlight (Caldwell, 1977; Tevini and Teramura, 1989). From these studies, it is now recognised that there are both direct and indirect effects of UV-B taking place at the whole plant level, especially under natural conditions.

Direct effects

Studies have shown that plants exhibit a tremendous variability in their sensitivity to UV-B radiation. Responses that do occur include changes in leaf secondary chemistry (flavonoid accumulation), alterations in leaf anatomy and morphology, reductions in net carbon assimilation capacity (photosynthesis) and changes in biomass allocation and growth (Musil, 1996). The direct UV-B action on plants that results in changes in form or function of plants appears to occur more often through altered gene activity rather than non-specific damage to DNA (Britt, 1997). These include both mechanistic damage to the photosynthetic apparatus and changes in growth and morphology which may reduce light interception and competitiveness. Direct effects include radiation-induced changes in photosynthesis, cell division and other life processes of direct importance to growth and development such as alterations in plant hormones or nucleic acids. These effects are observed after relatively short periods of irradiation, which could be hours or days (Bornman and Sundby-Emmanuelsson, 1995).

In addition to the direct effects listed above, DNA damage reactions and also adaptive responses, including the switching on of a range of defence mechanisms to afford protection against UV radiation, can be included. Damage to DNA has long been recognised as an important consequence of exposure to UV and the products formed as a result of damage to DNA, and the activity of the photolyase enzyme(s) involved in repair have been described (McLennan, 1987; Pang and Hays, 1991; Quaite *et al.,* 1992; Stapleton, 1992; Taylor *et al.,* 1997). These are the UV radiation-induced pyrimidine dimers, of which there are two major classes : the pyrimidine [6-4] pyrimidone photoproduct (6-4 product), and the cyclobutane pyrimidine dimer (CPD). A general approach for screening for mutants defective in DNA repair as a way of isolating the genes involved in the repair processes has been described (Britt, 1997). Clearly, some of the UV-B induced down-regulation of photosynthetic genes (Jordan *et al.*, 1992; Baker *et al.,* 1997; Britt, 1997) is not simply a consequence of non-specific damage to DNA, since other defence-related genes are up-regulated, and there is developmental variation in response to UV-B. Furthermore, protection under high light appears to be due to some component of photosynthetic activity rather than to photolyase activity.

The best studied direct UV protection mechanism is the differential production of pigments, especially flavonoids (Fohnmeyer *et al.* 1997). This type of response involves the stimulation of expression of particular genes by UV-B, implying specific UV-B light detection systems and signal transduction processes, which lead to the regulation of transcription. For example, the synthesis of UV-absorbing protective molecules such as flavonoids, hydroxycinnamic acids and related compounds is not a damage response and involves the stimulation of expression of particular genes (Jenkins *et al.,* 1997). The largest concentration of these pigments is located in the epidermis, effectively reducing the penetration of UV-B deeper into the mesophyll cells of the leaf (Bornman *et al.,* 1997), thus acting to screen out the UV-B. In these cases, the responses are likely to involve specific UV-B photoreceptors and signal transduction processes that lead to the regulation of transcription (Jenkins *et al.*, 1997).

It is evident that not all the effects of UV-B on plants involve macromolecular damage. As already stated, plants exhibit a wide range of responses to UV-B,

including physiological responses which help to protect them from damaging UV-B wavelengths (Tevini and Teramura, 1989; Stapleton, 1992). Understanding the molecular basis of perception and signal transduction is therefore likely to be of direct relevance to a scenario of global climatic change.

Indirect effects

Indirect effects are those mediated by radiation-induced changes in the plant environment, or changes in the plant which are of importance mainly in relation to other organisms. Consequences of indirect solar UV-B radiation are quite important, yet less predictable. They act through changes in the chemical composition and growth form of plants through changes in the abiotic environment (Caldwell *et al.*, 1998). Indirect effects include effects on other plants that compete with the plant under consideration, nutrient mobilisation, and on herbivores and micro-organisms of importance to the plant. Examples include changes in the susceptibility of plants to attack by insects and pathogens in both agricultural and natural ecosystems. The direction of those changes can result in either a decrease or increase in susceptibility. Other indirect effects include changes in competitive balance of plants and nutrient cycling. To understand the effects of UV requires the study of several components of natural ecosystems.

Unlike studies of direct effects, where responses occur quite rapidly, studies of indirect effects need to be carried out over a long period of time since effects are likely to be of a slow, cumulative nature. To date, more effort has been given to understanding the direct effects, and indeed these are probably the most important ones in crops, growing as they do in a partly human-controlled environment, and mostly in monoculture. Indirect effects however, may be more important for wild populations in a natural environment.

1.2.1 UV-B effects on plant systems and their responses

The potential impacts of an increase in the solar UV-B radiation reaching the earth's surface due to stratospheric ozone depletion have been investigated by several research groups during the past two decades (Caldwell *et al.*, 1998; Grammatikopoulos *et al.*, 1998; Strid *et al.*, 1994; Tevini and Teramura, 1989). In

these studies, UV-B radiation was found to be particularly detrimental to plants. Much of this research centred on the effects on plant growth and physiology under artificial UV-B irradiation supplied to plants in growth chambers or greenhouses. These studies however, were performed with non-realistic doses and spectral distributions of UV-B radiation, usually in growth chambers or glasshouses where, in addition, the natural balance between UV-B, UV-A and visible radiation was considerably altered. Possible anomalies in assessing the biological effectiveness of the irradiation sources and of predicted ozone depletion could occur because of the wavelength dependency of photobiological processes and the fact that the artificial sources do not precisely match the solar spectrum. To interpret results from such investigations, weighting functions based on action spectra for specific responses, have been developed.

The process by which light regulates aspects of plant growth is termed photomorphogenesis (Kendrick *et al.,* 1997). Some of the sub-processes that are regulated by light signals include changes in structure and form, such as seed germination, leaf expansion, stem elongation, flower initiation and pigment synthesis. These photomorphogenic responses confer an enormous survival advantage on organisms. However, relatively little is known about the types of photomorphogenic responses and signal transduction pathways that plants employ in response to UV-B radiation. What is known is that competitive interactions may also be altered indirectly by differential growth responses, while photosynthetic activity may be reduced by direct effects on photosynthetic enzymes, metabolic pathways or indirectly through effects on photosynthetic pigments or stomatal function (Baker *et al.*, 1997).

Overall, the effectiveness of UV-B varies both among species and among cultivars of a given species. Sensitive plants often exhibit reduced growth (plant height, dry weight, leaf area, *etc.,*), photosynthetic activity and reduced flowering. Most of these responses are mediated by a number of light-absorbing molecules that enable organisms to respond to changes in the natural light environment. Because UV-B has been implicated in the inhibition of plant growth and possibly mutations, studies are in progress to screen for mutants with enhanced sensitivity to either of these effects (Jenkins, 1997). It should be noted however, that mutants expressing either of these

UV-sensitive phenotypes might be defective in processes other than DNA repair, including defects in the production of UV-B absorbing pigments, defects in the ability to cope with a variety of stresses, or defects in other UV-sensitive processes such as photosynthesis. Through this approach, useful insights into the biochemical basis of UV-induced growth inhibition are likely to be gained.

Section 1.2.1.1 Changes in photosynthetic gene expression in response to UV-B

The impact of an increase in UV-B on the physiological parameters and morphological features of plants has been studied extensively (Bornman and Teramura, 1993). However, the knowledge of the effects of UV-B at the biochemical and molecular levels is limited. It is well established that a major site of damage by UV-B is the chloroplast, leading to impairment of photosynthetic function (Bornman, 1989). In the chloroplast, the integrity of the thylakoid membrane seems to be much more sensitive than the activities of the photosynthetic apparatus bound within. Recently, a few studies have focused on the molecular mechanisms underlying UV-B sensitivity of photosynthesis (Strid *et al.,* 1994; Baker *et al.*, 1997; A-H Mackerness *et al.,* 1999). Changes in gene expression reported in response to supplemental UV-B include reduction in expression and synthesis of key photosynthetic genes including Rubisco (*rbc*S and *rbc*L), D1 polypeptide of photosystem II (*psb*A), chlorophyll *a/b*-binding protein (*Lhcb* or *cab*), a decline in total RNA enzyme activity and the ATPase complex. The ATPase complex is involved in the hydrolysis of ATP to ADP and orthophosphate, as well as functioning as an exchanger or transporter for NA^+, K^+ or Ca^{2+} (Jordan, 1996; A-H Mackerness *et al.,* 1997b; Baker *et al.,* 1997). Decreases of mRNA transcripts for photosynthetic complexes and other chloroplast proteins have been reported as being among the very early events of UV-B damage, pointing to the effect of UV-B on photosynthesis as well as protein synthesis (Strid *et al.,* 1994). Other genes encoding defence-related enzymes e.g. of the flavonoid biosynthesis pathway, are rapidly up-regulated following commencement of UV-B exposure.

1.2.2 Perception and signal transduction of UV-B radiation

Light is probably the most dominant environmental factor that regulates plant development and plant gene expression. Without light, life as we know it would not

exist. Plants harvest solar energy by photosynthesis and provide energy to other organisms through the food chain. The numerous effects of light on plant growth and development are mediated by several classes of photoreceptors coupled to signal transduction networks (Jordan, 1996). To monitor light quality and quantity, and temporal and spatial patterns of light, plants have evolved at least three different photoreceptor systems, namely phytochromes, blue-light/UV-A photoreceptors and UV-B photoreceptors (Jenkins, 1997). The best-characterised photoreceptors are the phytochromes, which affect responses principally to red and far red light. Phytochromes are soluble pigmented proteins of approximately 125 kDa. The phototypical phytochrome is a homodimer, each subunit of which contains a covalently linked linear tetrapyrrole chromophore. Phytochrome mediates the responses to red and far-red light through its ability to photointerconvert between two stable isomers: a red light-absorbing form termed P_r (λ_{max} = 660 nm) and a far-red light absorbing form termed P_{fr} (λ_{max} = 730 nm) (Kendrick and Nagatani, 1991; Khurana *et al.,* 1998). Although phytochromes also absorb UV and blue light, it is well established that most of the responses of plants to this region of the spectrum are mediated by separate photoreceptors, absorbing specifically UV-A (320-400 nm) and blue (390-500 nm) wavelengths (Briggs and Huala, 1999). Examples of responses regulated by UV/blue light include: stem extension, chloroplast development, phototropism, stomatal opening, leaf expansion and the expression of various genes.

The UV-B photoreceptors are the least understood and are thought to mediate UV-B effects, even though their mode of action is presently unknown. All higher plants examined have multiple genes for phytochromes. In *Arabidopsis*, five different genes, termed *phy*A through *phy*E encode the apoprotein component of phytochrome. These phytochromes-encoding genes, together with two genes encoding blue/UV-A photoreceptors, are currently under investigation in *Arabidopsis thaliana* (Nakajima *et al.*, 1998).

The plasma membrane of plant cells undergoes a number of changes in response to UV-B exposure. These changes include an efflux of K^+ ions, depolarisation of the cell's electrical potential, synthesis of H_2O_2, and oxidation of reduced glutathione (GSH) to oxidised glutathione (GSSG) (Strid *et al.*, 1990). The changes to the plasma

membrane are considered to be induced in response to the UV-B and not a result of direct photochemical damage and subsequent loss of membrane integrity. Many different plant responses to supplemental UV-B radiation have been observed. These include biomass reduction, decreases in the percentage of pollen germination, changes in the ability of crop plants to compete with weeds, epidermal deformation, and changes in cuticular wax and increased flavonoid levels. These changes could result from any number of primary UV-B events, DNA damage, direct photosynthetic damage, membrane changes, protein destruction, hormone inactivation, signal transduction through phytochrome (which photoconverts in response to UV-B), or signal transduction via an UV-B photoreceptor.

Nuclear encoded genes are reportedly more sensitive than genes encoded by the chloroplast (Jordan *et al.*, 1992). The relative sensitivity of transcripts to UV-B radiation is also dependent on the developmental stages of the tissue studied. In contrast to the down-regulation of genes encoding photosynthetic proteins, UV-B irradiation results in the up-regulation of some defence genes such as chalcone synthase and glutathione reductase. Chalcone synthase (*chs*) is a key enzyme in the synthesis of flavonoids, which are produced under a variety of conditions, including UV-B exposure, and are thought to act as UV-B screening pigments. Glutathione reductase on the other hand, is thought to be an important part of a system designed to scavenge active oxygen produced in response to oxidative damage caused by exposure of plants to a multitude of stresses. This is essential for survival and for prevention of genetic diseases since unrepaired or improperly repaired lesions can be cytotoxic and mutagenic. These damages are initiated by a wide variety of environmental and endogenous agents, particularly reactive oxygen species (ROS) that are generated spontaneously during respiration.

An increase in the level of mRNA for these genes indicates that repression of gene expression for chloroplast proteins is a specific response to UV-B treatment and not a result of non-specific damage to DNA. Studies of the effects of UV-B on mRNA levels indicate that UV-B mediated inhibition of photosynthetic performance can, to some extent, be related to decreases in levels of photosynthetic proteins. However, they do not show whether the effect of UV-B arises through changes in transcription, translation or post-translational events (A-H Mackerness *et al.*, 1997a,b). The

accumulation of flavonoid compounds in response to UV-B has been shown to be due to an increase in the rate of transcription of the chalcone synthase gene. However, the level at which UV-B down-regulates the genes encoding for the chloroplast proteins has not been studied.

Because plants are constantly exposed to solar radiation, the consequences of any increase in UV-B radiation are likely to be most obvious in their effect upon plant growth and development (Caldwell, 1981; Teramura, 1983; Tevini and Teramura, 1989). Individual plant cells have the capacity to respond to a wide range of signals that regulate their growth and differentiation and affect their survival. A key point is that the cells must have mechanisms that allow signals to be detected and acted upon to give rise to particular responses. For instance, to enable the UV-B radiation to have an effect, the plant must first absorb it. The plant may perceive the UV-B radiation by a specific mechanism involving photoreceptor molecules or by non-specific absorption by other cellular constituents (Frohnmeyer *et al.*, 1997; Jenkins *et al.*, 1995; Jenkins, 1997). The detection of signals in many cases is likely to involve specific cellular components termed receptors, and reception is coupled to the terminal response by signal transduction mechanisms. Once the plant perceives the UV-B radiation, the information must be transmitted through cells or tissues to target sites where it may elicit a response. This transmission is frequently referred to as the signal transduction pathway and is composed of the secondary messenger(s), an amplification mechanism and the responsive component within the cell. To date, mainly biochemical approaches have been used to study UV-B and blue/UV-A signal transduction pathways (Lois and Buchanan, 1994). A major advance was the cloning of the *Arabidopsis* CRY1 (cryptochrome) photoreceptor encoded by the *hy*4 gene (Ahmad and Cashmore, 1993, 1996). CRY1 mediates some responses to UV-A, blue and green light. Mutants in the *hy*4 gene are impaired in the suppression of hypocotyl extension by these wavelengths, and several other extension growth responses. In addition, *hy*4 mutants have reduced induction of flavonoid biosynthesis gene expression and anthocyanin synthesis in blue light (Jenkins, 1997). CRY1 is postulated to regulate the extension growth and gene expression responses through separate or branching signal transduction pathways.

Studies of the CRY1 protein indicate that it binds flavin and pterin chromophores, pointing to the possibility of electron transport being an initial event in CRY1 signal transduction. Evidence that membrane processes are involved in signal transduction is also available. For instance, calcium fluxes appear to be part of the signalling events which couple CRY1 to the regulation of transcription (Christie and Jenkins, 1996). In addition, membrane potential changes and ion fluxes are associated with blue light–induced extension growth responses. Furthermore, these blue light induced membrane potential changes and H^+ fluxes are associated with stomatal openings in several species.

It is quite clear that the successful existence of all higher organisms is dependent upon their ability to co-ordinate complex developmental changes and to sense and respond to fluctuations in their surroundings, and this process occurs by stimulus-response coupling. Key properties of signal transduction are therefore, speed, sensitivity (achieved by amplification), and specificity, all of which are controlled by a network of positively and negatively acting elements (Bowler and Chua, 1994). Specificity of the response for the stimulus is particularly important for genetic studies because in many cases it is desirable to generate mutants that are constitutive for a response in the absence of the stimulus. This is meant to ensure that all of the phenotypes of the mutant are consistent with all, or at least a subset of, the normally observed phenotypes of wild-type plants exposed to the stimulus.

1.3. General protection mechanisms against UV-B radiation

Plants are constantly exposed to ultraviolet radiation that is present in the spectrum of solar radiation due to their static lifestyle and obligatory requirement of light for photosynthesis. Plants occupy a special niche owing to their ability to obtain energy directly from sunlight and as a result, have acquired tolerance mechanisms during evolution and development. They also have a range of mechanisms to protect themselves against UV-radiation damage and to limit the amount of DNA damage resulting from solar UV-B radiation (Taylor *et al.,* 1997). They employ several mechanisms which essentially involve two basic strategies, either shielding or repair. A well-characterised response of plants to UV-B radiation is the synthesis of protective pigments that absorb short-wave radiation and prevent that radiation from

entering their cells (Jordan, 1996). A vast majority of higher plants exhibit the characteristic accumulation of UV-absorbing pigments, notably flavonoids 9described in detail in section 1.4), in the upper epidermal layers following irradiation with UV-B. These phenolic compounds attenuate the damaging UV-B radiation but transmit photosynthetically active radiation (PAR) to the underlying palisade and mesophyll tissue where the bulk of photosynthetic reactions take place. For example, in maize UV-B absorbing flavonoid pigments have been implicated in UV-protection (Stapleton and Walbot, 1994), whereas sinapic esters which are biosynthetic precursors of lignin may be particularly important as sunscreens in *Arabidopsis* (Landry *et al.,* 1995). However, sunscreens are not completely effective and plants must also have mechanisms that enable them to cope with the cellular damage caused by the UV radiation that is able to penetrate the cells. To mitigate the effects of UV-induced DNA damage on transcription and replication, all organisms exhibit a range of DNA damage control strategies, and many of these repair mechanisms appear to be broadly conserved.

1.4 Flavonoids biosyntheses and their role in plant DNA protection

1.4.1 Flavonoids and anthocyanins

Rapid protective response to the damaging effects of UV irradiation is paradigmatic of active defence mechanisms in plants. In many cases protection is thought to derive from the induced accumulation of strongly UV-absorbing flavonoid compounds in the outer tissue layers, preferentially in epidermal layers, which presumably protect sensitive targets from UV-damage (Schulze-Lefert *et al.*, 1989). Most higher plants accumulate UV-B absorbing pigments in their leaves particularly phenylpropanoids such as cinnamoyl esters, flavones, flavonols, and anthocyanins esterified with cinnamic acids after irradiation with UV-B (Wellmann, 1983). In addition to phenyl propanoids, other important products of the shikimic acid pathway such as furanocoumarins, and polyketides and terpenoids such as canabinoids, also accumulate under increased UV-B radiation. Several researchers have found key enzymes in the biosynthetic pathways of these compounds to be specifically induced by UV-B irradiation (Schulze-Lefert *et al.*, 1989; Stapleton, 1992; Middleton and Teramura, 1993; Kootstra, 1994).

For plants, light is an essential source of both energy and external signals regulating developmental processes and adjustments to changes in the environment. In tissues exposed to potentially noxious UV-irradiation, a major form of adjustment is the rapid accumulation of UV-protective compounds (Schemelzer *et al.*, 1988). Flavonoids accumulate in the vacuoles of epidermal cells and absorb strongly in the critical range of 230-380 nm where damage caused by UV-irradiation occurs. They are synthesised in vascular plants in response to a variety of environmental stimuli and are reported to play a role in protecting plants from the deleterious effects of UV-radiation (Beggs *et al.*, 1985). This is because flavonoids such as flavones, and also anthocyanins, which have been observed to be induced by visible light in a great variety of plant species, absorb light in the UV-region (Beggs and Wellmann, 1994; Buchholz *et al.*, 1995). Flavonoids could thus play a role in protecting against UV-induced DNA damage in plants (Stapleton, 1992; Middleton and Teramura, 1993; Kootstra, 1994).

It has been shown that flavonol accumulation is specifically UV-induced and is linearly dependent on UV-B fluence (Wellman, 1983). This increase in flavonoid concentration is due to a higher activity of the key enzyme PAL (phenylalanine ammonium lysase) and/or to higher rates of biosynthesis of this enzyme (Tevini and Teramura, 1989). The flavonoids are derivatives of cinnamic acid, which is formed through the deamination of phenylalanine. The first steps in the conversion of phenylalanine to derivatives of cinnamic acids are common to the pathways for the biosynthesis of cinnamate esters, flavonoids and lignins; these early steps are known as the general phenylpropanoid metabolism. Biosynthesis of various flavonoids from phenylalanine is brought about via transcriptional activation of genes of phenylpropanoid metabolism. The biosynthetic pathway of all classes of flavonoids is initiated by the enzyme chalcone synthase (CHS) (Heller and Forkmann, 1988; Mol *et al.*, 1996). CHS and other enzymes involved in biosynthesis serve as model systems for a better understanding of molecular aspects of gene regulation by light as these pigments have an important function against damaging effects from shorter wavelength solar radiation.

The phenylpropanoid pathway, which is unique to plants, catalyses the conversion of phenylalanine to a myriad of phenolic secondary metabolites including lignins,

sinapate esters, stilbenes, and flavonoids, the latter of which also incorporate carbon from malonyl-CoA. These compounds play diverse roles in essential plant processes, including growth, defence, protection from UV light and reproduction (Shirley, 1999). In addition to their probable role as UV protectants, diverse structural classes of flavonoids serve ubiquitous roles as flower or fruit pigments and potential insect repellents or attractants. The enzymes that catalyse the individual steps are: phenylalanine ammonia-lyase, cinnamet-4-hydroxylase, and 4-coumarate:CoA ligase. A reaction between 4-coumaroyl-CoA and malonyl-CoA, catalysed by the enzymes flavanone synthase (chalcone synthase) and chalcone isomerase, results in the formation of naringenin, a flavone with the basic $C_6C_3C_6$ structure of flavonoids. The flavonoids, which are rather abundant, share a common structural unit, the $C_6C_3C_6$ skeleton of flavone and are widespread throughout the plant kingdom. It has been estimated that about 1.5% of the carbon fixed annually in photosynthesis is used for the synthesis of flavonoids. It is uncertain if naringenin or a chalcone isomer of it is the precursor for the successive steps in the synthesis of flavonoids. The steps involved in the biosynthesis of flavonoids have been summarised in several articles (Grisebach, 1979; Hahlbrock and Grisebach, 1979; Wong, 1976).

The anthocyanins (glycosylated anthocyanidins) are water-soluble, vacuolar pigments, responsible for the violet, blue, purple, dark red and scarlet coloration of fruits, flowers, stems and leaves in all orders of higher plants. One exception is the order *Centrospermae*, where the violet-red colours are due to a different group of water-soluble vacuolar pigments, the flavonoids. These include flavonols, flavones, flavanones, cathochins, chalkones and others in addition to the anthocyanins. The capability for the formation of anthocyanin is determined by hereditary factors whereas the amount of pigment formation is affected by numerous environmental factors such as nutritional and waters conditions, wounding, infections, age, temperature and light.

1.5 Effects of UV-B on DNA

1.5.1 DNA damage

Environmental and physiological studies have been conducted to ascertain whether ambient and enhanced solar UV-B levels retard growth, development and biomass accumulation in plants (Musil and Wand, 1993, 1994; Musil, 1994, 1995; Strid *et al.*, 1994). The effects of elevated UV-B radiation on plants were found to vary widely among species and even among cultivars. These effects range from the molecular scale, such as DNA damage, to tissue and whole plant effects, including decrease in photosynthetic activity and changes in plant structure and biomass (Caldwell *et al.*, 1989; Harlow *et al.*, 1994, and Musil, 1994). Concern over these potential effects has prompted several studies by environmental photobiologists geared towards elucidating effects of possible genetic and ecological consequences of increased UV-B radiation.

Radiation and some chemical agents frequently produce modified bases within DNA that prevent accurate replication or transcription (D'mitry *et al.*, 1995). For example, ultraviolet radiation UV-B and or UV-C gives rise to a multitude of DNA photoproducts (Sancar and Sancar, 1988; Taylor *et al.*, 1997). The most common DNA photoproducts induced by UV-radiation are cyclobutane dimers between two adjacent pyrimidines (TT, CT, TC, or CC), and pyrimidine (6,4) pyrimidone, also formed by adjacent pyrimidines. Biological effects of these lesions have been studied extensively in mammalian and microbial systems where the UV-B induced damage to DNA has been shown to lead to either mutagenesis or toxicity (Britt, 1997). These damaged DNA molecules may cause mutations if replicated (Jiang and Taylor, 1993), thus repair of UV-B radiation-induced damage to DNA is important for all living organisms, especially plants. However, very few studies with plants have been directed toward the identification of DNA photoproducts, subsequent mutations and repair mechanisms (Stapleton, 1992; Strid *et al.*, 1994; Kootstra, 1994; Britt, 1995; Buchholz *et al.*, 1995). At the molecular level, pyrimidine dimers are known to inhibit the progress of microbial and mammalian DNA polymerases. The formation of these DNA photoproducts gives rise to changes in the base-pairing properties between two DNA strands at the site of the lesion. The change in base-pairing

properties is most notable for the cytosine (C)-derived pyrimidine adducts and results in a high degree of adenosine (A) incorporation into the new complementary DNA strand during replication. With TT photoproducts, such misincorporation occurs only rarely. Because pyrimidine dimers cannot effectively base pair with other nucleotides, they are not directly mutagenic, but instead act as blocks to DNA replication (Britt, 1997). Thus a single pyrimidine dimer, if left unrepaired, is sufficient to completely eliminate expression of a transcriptional unit. When the newly formed strand, with its substituted nucleotides, later serves as template for further replication, C=>T mutations or CC=>TT tandem mutations occur. In addition to the two most common UV-induced photoproducts already mentioned, other types of DNA photoproducts exist, such as purine containing photoproducts, hydration of pyrimidines, insertion or deletion of base pairs resulting in frame-shifts, DNA strand breaks and cross-linking of DNA to proteins.

Although the detailed mechanism by which biological effects of UV are produced and the effects that such increased UV-B exposure might have on plant life at the molecular level is largely unknown, the evidence is overwhelming that changes in DNA such as formation of pyrimidine dimers and other photochemical products, have important biological consequences (Stapleton, 1992; Kootstra, 1994; Strid *et al.*, 1994; Britt, 1995). Despite the lack of thorough characterisation of the biological effects of these lesions, they are generally assumed to be similar to those observed in other living kingdoms. In many cases, for microbial systems, it has been possible to correlate the production of specific photoproducts in DNA with biological changes such as the inactivation of biologically active DNAs, the killing of cells and the induction of mutations (Setlow and Setlow, 1972; Setlow 1974).

The formation of different photoproducts upon UV-B radiation exposure damages DNA molecules and eventually blocks DNA replication and transcription in plants cells (Britt, 1997). This is because DNA is a highly reactive molecule that is sensitive to damage from a wide range of both physical and chemical agents (making it an especially vulnerable target for UV-induced damage), as well as being considered to be the primary absorbing chromophore in the cell in the UV-B region of the spectrum. If every pyrimidine dimer acts as a block to transcription and replication, while only a small fraction of dimers result in a mutation, the inhibitory effects of UV on

transcription and DNA replication are probably more significant in terms of plant growth than its mutagenic effects. Even a single persisting UV-induced lesion can be a potentially lethal event, particularly in haploid tissues such as pollen grains (Britt, 1997). However, a variety of mechanisms for repairing or circumventing the damage are present in both prokaryotes and eukaryotes, and the genes that regulate these mechanisms are regulated by DNA damage (see section 1.5.2, DNA repair).

Nevertheless, studies suggest that high, but not excessive, irradiance at the visible wavelengths, before or during exposure to UV-B may ameliorate the inhibitory effects of UV-B exposure on plant growth. Possibly, one of the effects of increased irradiance of visible light during UV-B exposure is to help protect normal gene expression. The cyclobutane pyrimidine dimer and the pyrimidine (6-4) pyrimidone dimer (the 6-4 photoproduct) make up the bulk of UV-induced DNA damage products. Unrepaired dimers are lethal to cells because they deform the DNA helix, interfering with both replication and transcription. These biological effects are significantly reduced by subsequent exposure to light, a process known as photoreactivation (Sakamoto *et al.*, 1998). Thus it is important to study DNA damage and repair mechanisms in plants, particularly since plants have an obligatory requirement for sunlight. The molecular analysis of DNA repair mechanisms depends on the assay of pyrimidine-containing dimers as a result of radiation exposure, and their removal or tolerance in the DNA at various stages of the repair process.

1.5.2 DNA repair

The UV component of sunlight produces cytotoxic, mutagenic and carcinogenic lesions in DNA. In order to prevent alterations in their genetic information due to DNA damage, organisms have efficient mechanisms of DNA repair and recombination (Britt, 1995, 1996; Cerutti *et al*, 1993). The evolution of various repair mechanisms to counteract the deleterious effects of UV radiation on cellular DNA has led to a number of studies aimed at elucidating these repair processes. The most important discovery in this area of research in recent years is that all cells have a remarkable capacity to repair damage that is produced by UV in their DNA. The most detailed studies have been carried out on *E. coli*, yeast and mammalian cells (Sancar and Tang, 1993). Based on these studies, cells appear to have evolved a

variety of biochemical mechanisms to restore the integrity and retain the stability of the genetic material after DNA damage. These processes, defined as DNA repair, are organised into a number of pathways that are functionally distinguished by the type of chemical modification which they remove (Taylor *et al.*, 1997). The systems of repair responsible for the removal of damage by UV-B radiation from cellular DNA include photoreactivation (PHR), excision repair, including nucleotide excision repair (NER) and base excision repair (BER), recombinational repair and post replication repair (Sancar and Sancar, 1988). Our knowledge of DNA repair mechanisms in plants lags far behind our understanding of these pathways in microbial and mammalian systems in which most of the mechanisms have been elucidated (Britt, 1995).

Photoreactivation

Although a substantial number of DNA photoproducts are probably formed during UV-exposure, these can in turn be counteracted by specialised photoreactivating enzyme systems such as photolyases. Photoreactivation is the prevention of the deleterious effects of far-UV light (200 to 300 nm) by concurrent or subsequent exposure to near UV-visible light (300 to 500 nm) (Hearst, 1995). Photolyases are flavoproteins that contain two noncovalently bound flavin chromophores. The photoreceptor chromophore, which is usually either deazaflavin or folate, binds to an N-terminal region of the protein. All known DNA photolyases are single polypeptide enzymes that contain reduced FAD and a second chromophore which is either methyltetrahydrofolate or a deazaflavin, depending on the source of the enzyme. The C-terminal portion of the apoprotein binds fully reduced flavin adenine dinucleotide ($FADH^-$), which mediates the photoreduction of CPDs and (6-4) photoproducts (Hearst, 1995; Taylor, 1997; Taylor *et al.*, 1997).

Many organisms, including plants contain direct-acting light-dependent DNA-photolyase enzymes that specifically bind to UV damage products and split them by breaking the cyclobutane ring via a UV-A/blue light-dependent mechanism, thereby reversing the damage in an error-free manner, and preventing mutations. Photolyase utilises photons of light in the UV-A or blue light region of the spectrum (300-500 nm) as an energy source to monomerise dimers by a process involving photoinduced electron transfer (Sancar, 1994). During photoreactivation repair, the N-terminal

flavin absorbs light of wavelengths in the region 375-445 nm and transfers the excitation energy to the C-terminal $FADH^{\cdot}$, which donates an electron directly to the dimer. The resulting electrochemical rearrangement restores the pyrimidine bases to their normal structures and the electron is returned to the $FADH^{\cdot}$ radical (Lin *et al.*, 1996; Sancar, 1996).

The light-dependent repair enzyme, photolyase was originally reported to be responsible for the direct splitting of pyrimidine cyclobutane dimers but not pyrimidine(6-4)pyrimidone photoproducts. Recent reports have demonstrated that photoreactivation of UV-induced dimers occurs in higher plants, including photoreactivation of (6-4) products as well as CPDs (Pang and Hays, 1991, Nakajima *et al.*, 1998). Pang and Hays (1991) demonstrated the presence of UV-B induced cyclobutane dimer formation, dark repair and photoreactivation in *Arabidopsis thaliana*. The *A. thaliana* photolyase was induced by UV-B exposure but UV-A radiation was most effective for photoreactivation. The need for determination of action spectra for DNA damage in more plant species cannot be overstated as it would produce a generalised plant response curve incorporating most wavelengths of UV-radiation.

Photolyases are thought to be critical components of the defence of plants against damage to DNA by ultraviolet light. A photolyase gene from mustard was recently cloned (Batschauer 1993). The gene encodes a polypeptide of 501 amino acids with a predicted molecular mass of 57 kDA and shows a strong sequence similarity to bacterial and yeast photolyases, and a close relationship to enzymes with a deazaflavin chromophore. The plant photolyase was shown to be functional in *E. coli* pointing to conservation of photolyases during evolution. The fact that photolyase expression in plants was light-inducible provided good evidence for the adaptation of plants to their environment in order to diminish the harmful effects of sunlight.

The genes encoding the CPD photolyase are widely distributed amongst species, and have been characterised in detail (Nakajima *et. al* 1998). For example, light–enhanced repair of dimers from total DNA has been documented in tobacco, *Haplopapus gracilis,* ginkgo, *Chlamydomonas, Arabidopsis* and wheat. The action spectrum for reversal of CPDs by partially purified maize and *Arabidopsis*

photolyases has been shown to be similar to that of *E. coli* (Britt, 1996). Light-dependent repair of 6-4 photoproducts has been observed *in vitro* in the higher plant *A. thaliana.*. A gene that has sequence similarity to a gene that expresses a protein with a 6-4 photolyase activity *in vitro* in *Drosophila. melanogaster* and *Xenopus laevis* was recently cloned from this plant. This gene (called UVR3) produced a protein with 6-4 photolyase activity when expressed in *E. coli.*

Excision repair

Excision repair can repair a range of damage to DNA including dimers. DNA damage, photoreactivation and excision repair in the dark have been reported in higher plants but mainly following UV-C exposure (Soyfer and Creminis, 1977; Strid *et al.,* 1994). In contrast with photoreactivation, dark repair pathways do not directly reverse DNA damage. Instead they replace the damaged DNA with new, undamaged nucleotides through excision repair pathways, namely nucleotide excision repair and base excision repair. Excision repair processes are generally referred to as "dark repair" processes to distinguish them from other light-dependent DNA repair processes.

*Nucleotide excision repair (*NER)

Nucleotide excision repair is considered to be the second major pathway for removal of UV photoproducts from cellular DNA. It is also the most versatile strategy for the repair of DNA damage, responding to a diverse range of both chemical and photochemical lesions, including the UV-induced CPD and (6-4) photoproduct lesions (Taylor *et al.*, 1997). NER was first elucidated in *E. coli* and acts on lesions that have created significant distortions in the DNA double helix (including pyrimidine dimers and (6-4) photoproducts), and incorporates a multisubunit endonuclease which incises a segment of single-stranded DNA encompassing the lesion.

It involves a five-step pathway that can remove a variety of unrelated helix-distorting bulky lesions by replacement rather than the direct reversal of damage. The sequence of events involves recognition of the damage, local unwinding of the DNA by a helicase action, endonucleolytic incision and removal of an oligonucleotide

containing the damage. This is followed by resynthesis of a stretch of DNA using the complementary strand as template and ligation of the newly synthesised DNA to the existing chain (Friedberg *et al.*, 1995). This process is widespread amongst species from bacteria through to mammals (Taylor *et al.*, 1997). Few investigations of NER in plants have been reported, but light-independent "dark" repair of CPDs, which might represent either NER or base excision repair, has been observed in several plant species (Britt, 1996; Liu *et al.*, 2000, Vonarx *et al.*, 1998; Xu *et al.*, 1998). The rate of dark repair of CPDs was found to vary widely between plant species. High rates of repair were demonstrated for carrot suspension cultures and carrot protoplasts, *Haplopappus*, petunia, and tobacco, whereas excision repair of CPDs was undetectable in cultured soybean cells.

DNA repair studies in *Arabidopsis* indicated that the removal of CPDs in the dark proceeded at a rate lower than that of photoreactivation. However, repair of (6-4) photoproducts in the absence of light has been reported in *Arabidopsis*, in which the UV-sensitivity of one mutant was determined to be due to defective dark repair of (6-4) photoproducts (Vonarx *et al.*, 1998). Since photoreactivation is mainly active in visible light the process involved in repair in the above study is therefore most likely NER since it has been reported to repair both CPD and (6-4) photoproducts lesions. Several genes involved in the NER pathway and their homologues have now been cloned, e.g. a rice *RAD23* structural homologue, a gene involved in transcription control was recently sequenced (Schultz and Quatrano, 1997). In addition, several putative *RAD23* homologues are reported to be present in the *Arabidopsis* expressed sequence tags (EST) databases. For example, two *Arabidopsis DRT101* and *DRT102* cDNAs were found to partially complement the UV-sensitivity of an *E. coli* mutant and were suggested to encode UV-specific excision repair enzymes (Pang *et al.*, 1993).

Base excision repair (BER)

BER is an excision repair process that may be less important with regard to UV damage repair, but nevertheless excises modified bases from the genome and replaces them with normal nucleotides. This process can proceed through various enzymatic stages. BER consists of a collection of damage-specific DNA glycosylases which

remove modified bases from DNA in a highly specific and low energy cost to the cell process, giving BER unique features. The damaged bases are cleaved by the action of DNA glycosylases, which catalyse the cleavage of the N-glycosylic bonds between the modified bases and their sugar moieties, leaving the DNA sugar-phosphate backbone intact (Britt, 1996). This reaction leaves abasic sites within the DNA, which are then removed by apurinic/apyrimidinic (AP) endonucleases or AP lyases, which nick the backbone of the DNA at the AP site. The nicked DNA is then restored to its original sequence through the combined actions of exonucleases, a repair polymerase, and DNA ligase. These endonucleases specifically catalyse the hydrolysis of phosphodiester bonds at abasic sites in the DNA (Friedburg *et al.*, 1995). Apparently, this pathway evolved to protect the cell against effects of endogenous DNA damage, but the pathway also appears to be essential for the resistance to DNA damage from exogenous damaging agents such as UV-B. BER is known to be involved in the repair of minor UV-induced photoproducts such as thymine glycols which are a direct but minor product of UV-B irradiation (Taylor *et al.*, 1997). Recently, an *Arabidopsis* DNA ligase reportedly involved in excision repair (by homology to eukaryotic endonucleases) was cloned and sequenced (Taylor *et al.*, 1996). The formation of AP sites in maize seeds attributable to the action of DNA glycosylases on lesions accumulated during seed storage has also been reported (Vonarx *et al.*,1998), suggesting the existence of base excision repair in *Zea mays*. The presence of other glycosylases have been reported in cultured *Daucus carota* cells, as well as in an *Arabidopsis* λYES library.

It has been suggested that the relative contributions of the pathways involved in repair depend upon initial levels of damage incurred to the DNA (Quaite *et al.*, 1994a). For example, at higher levels of damage to alfalfa seedlings, both types of repair made significant contributions to the removal of CPDs, but at lower damage levels, only photoreactivation could be detected. Thus it seems that plants have the capability to excise UV-photoproducts but in the removal of CPDs, photorepair may be favoured. It is conceivable however, that some of the lesions produced by UV light may not be substrates for a photolyase-type repair mechanism, and in these cases excision repair may be the only method of removal of these photolesions (Taylor *et al.*, 1997; Quaite *et al.*, 1994b).

The repair pathways described above are essentially error free. If, however, a cell undergoes DNA replication before repair is complete, a "non-informational" DNA ramage product, such as a pyrimidine dimer, will act as a block to DNA replication. DNA polymerase will normally reinitiate synthesis 3' to the lesion, but a gap remains in the newly synthesized daughter strand at the site opposite the DNA damage product (Britt, 1996). Although one would expect the persistence of such a lesion to be lethal, a variety of organisms have been shown to undergo repeated rounds of DNA synthesis and cell division inspite of the continued presence of non-informational lesions. Translesion synthesis permits DNA replication, and therefore, enhanced survival, at the expense of accuracy. One example of such an independent pathway permitting the completion of replication of damaged chromosomes is recombinational repair. This pathway is thought of as a "damage tolerance pathway" because it does not involve DNA repair but instead helps the cell to survive despite persisting damage (Britt, 1996).

Recombinational repair

DNA double-strand breaks (DSBs) on chromosomes may arise during DNA replication, cleavage by site-specific endonucleases, or due to exogenous factors such as ionising radiation or chemical DNA-damaging agents (Michel *et al.,* 1997; Haber, 1999). DSBs are key intermediates in recombination reactions of living organisms and play an important role in homologous recombination in eukaryotes (Puchta *et al.,* 1996). Induction of DSBs has been shown to increase the frequency of homologous recombination. Since DSBs represent a lethal lesion, all organisms have developed repair pathways to correct such errors. Double strand breaks in DNA can be repaired in several ways and the most economical is ligation with another available DNA end. The two broken ends may be stuck back together by a process called non-homologous end-joining (also called illegitimate recombination) or the break may be repaired through genetic exchange with a homologous chromosome (homologous recombination). This dark repair system, also termed post-replication or recombinational repair, takes place after normal DNA replication (Caldwell, 1981; Vonarx *et al.,*1998; Van Dyck *et al.,* 1999). In this case, the undamaged portions of the DNA strand undergo normal replication leaving an appropriate-sized gap in place

of the lesion, which is then filled by DNA polymerase after initiating new DNA synthesis using the redundant information in the cell. Homologous recombinational repair usually proceeds by gene conversion, in which new DNA is copied from a donor template. It is of great importance that cells recognise the DNA DSBs and act upon them rapidly and efficiently, because major deleterious consequences can result if these are left unrepaired or are repaired inaccurately.

It should be noted that at present, very little is known about the genes required for illegitimate recombination in plants, but some *Arabidopsis* mutants displaying sensitivity to ionising radiation have been isolated (Britt, 1996). In *E. coli*, UV-photoproducts that are not repaired or removed prior to DNA replication may be "tolerated" by *recA*-dependent translession synthesis or post-replication mechanisms, and similar tolerance mechanisms have been reported in yeast involving members of the *RAD6* epistasis group for repair of UV-damage (Vonarx *et al.,* 1998). Genes encoding these post-replication homologues have now been isolated from wheat and *Arabidopsis.* For example, two cDNAs (*DRT111* and *DRT112)* for genes potentially involved in homologous recombination were isolated and they are postulated to play a role in DNA repair and /or recombination in *Arabidopsis.*

In a recent paper (Ries *et al.,* 2000a), elevated UV-B radiation was reported to reduce genome stability in plants. Elevated solar UV-B doses increased the frequency of somatic homologous DNA rearrangements in *Arabidopsis* and tobacco plants. The authors analysed the recombination frequency in somatic (non-reproductive) cells in response to natural spectral tolerance and also when UV-B was raised to higher levels. The progeny of plants exposed to high UV-B levels had a higher somatic mutation rate than their predecessors, and elevated UV-B was found to reduce genome stability in *Arabidopsis thaliana*, suggesting that plants were undergoing heritable and cumulative changes in the expression of genes involved in DNA metabolism.

Even though UV-B has been found to cause changes mainly in somatic tissue that is not going to transmit genes to the next generation, Ries *et al.*, (2000a) reported UV-B induced recombination in the reproductive (germ) cells, pointing to the likelihood of permanent changes in plant populations as a result of increased UV-B radiation levels. Plants showed somatic recombination in response to ecologically relevant increases in

UV-B radiation, and large elevations in UV-B radiation caused further genomic instability. In the same study, increases in recombination were accompanied by strong induction of expression of genes putatively involved in major DNA repair pathways (photoreactivation and recombination repair), namely photolyase and Rad51. This study presented intriguing data suggesting that elevated UV-B exposure over several generations may lead to progressive increases in somatic recombination rates as well as to higher numbers of permanently altered plants. An interesting observation was that effects of UV-B on genomic instability increased with each generation, suggesting that plants are undergoing heritable and cumulative changes in the expression of genes involved in DNA metabolism.

The strategies employed for the removal of DNA lesions show a high degree of evolutionary conservation between micro-organisms and humans, and CPD product repair in most organisms is due in large part to different combinations of the repair processes already described. Only recently have investigations focused upon DNA repair mechanisms in plants where it is assumed that similar mechanisms to those in other organisms operate. The precise mechanism of the DNA excision repair, which is prevalent from prokaryotes to eukaryotes, remains unknown, although a full understanding of its molecular basis is crucial for cell biology and the medical sciences.

1.6 *Dimorphotheca sinuata* and UV-B radiation

1.6.1 Distribution of *D. sinuata*

D. sinuata is an arid environment winter ephemeral of the family Asteraceae that grows in areas rich in other ephemeral species which differ in geographic distribution, leaf polyphenolic content and pollen type. Its range extends between 33°56'S, 18°29'E (Cape Town, South Africa) (see Figure 2.0) its southerly distribution, and 26°38'S, 16°18'E (Aus, Namibia) the northerly distribution limit where the anticipated stratospheric ozone depletion is 20% (Musil and Wand, 1993; Musil 1995). The plants are found in abundance on soils of variable fertility in areas

with unpredictable rainfall at lower latitudes in the north western Cape (van Rooyen *et al.*, 1990).

1.6.2 Protection by plant structures

The maintenance of genomic integrity is crucial to cellular survival. The genomes in a developing plant's reproductive organs seem better screened from UV-B radiation than in the leaves (i.e. shielding of the germline). For example, anther sacs shield male gametes by attenuating UV-B radiation by at least 98%, and the relatively thick ovary wall in most seeds protects female gametes well (Caldwell, 1981). Low UV-B transmitting perianth tissues, that also contain a variety of pigments (flavonoids, expoxy-carotenoids and xanthophylls) that absorb in the near UV-region, shield both. Dicotyledonous Asteraceae possess trinucleate pollen and high polyphenolic levels (Wand, 1995). *D. sinuata* forms seeds with two distinct morphologies : the ray morphs which have thick seed coats believed to protect seeds during conditions which are not conducive to germination, and the disk morphs which are lighter and aid in dispersal (C. Musil, personal communication). These two distinct morphologies could possibly play a role in shielding the seeds from the damaging effects of UV radiation during the time when they may lie exposed on largely barren soil surfaces for months and even years between rainfall events.

1.6.3 Physiological and biochemical effects of cumulative UV-B exposure on *D. sinuata*

Physiological and biochemical effects of cumulative exposure of *D. sinuata* to UV-B have already been studied *in vitro* (Musil and Wand, 1993; Musil, 1995, 1996; Midgley *et al.*, 1998). Data from these studies point to the possibility that UV-B effects could be cumulative over the life history of the study plant. The studies also showed that accumulated UV-B effects had a greater impact on plant performance than immediate UV-B effects (Musil, 1994, 1995).

Table 1.0. Summary of findings of prolonged UV-B exposure effects on offspring of *D. sinuata* from previous studies (see Musil 1995, 1996; Musil *et al.*, 1999a).

Attributes of offspring of progenitors grown under elevated UV-B

- Diminished photosynthetic rate – a consequence of a reduced leaf density
- Diminished foliar levels of carotenoids, polyphenolics and anthocyanins
- Substantial reductions in drymass
- Decreased stem and inflorescence production
- Earlier reproductive effort
- Diminished steady state fluoresence yields
- Diminished chlorophyll concentrations
- Diminished pollen tube growth and germination of seed set
- Increased non-structural carbohydrates
- Increased chlorophyll-*b* levels

Altered physiology was accompanied by reduced apical dominance and earlier flowering – features generally considered under photomorphogenic control, increased branching and inflorescence production and greater partitioning of biomass to reproductive structures, but diminished seed production. Comparisons with earlier generations revealed trends with cumulative generations of enhanced UV-B exposure of increasing chlorophyll-*b* and non-structural carbohydrates, decreasing polyphenolics and biomass allocation to vegetative structures, and diminishing seed production despite increasing biomass allocation to reproductive structures (Musil *et al.*, 1999a).

Changes brought about by accumulated UV-B included earlier reproductive effort, substantial reductions in dry mass, decreased stem and inflorescence production, diminished steady-state fluorescence yields, chlorophyll-*a* concentrations, pollen tube growth and germination of seeds set. On the other hand, immediate UV-B effects caused only diminished non-photochemical quenching, reduced concentrations of

chlorophyll-*a*, soluble sugar and starch, decreased pollen germination and increased carotenoid contents (Musil, 1996). The effects of UV-B irradiation on growth and allocation of biomass appeared to accumulate as subsequent generations were exposed to UV-B irradiation. Furthermore, after four generations of UV-B irradiation, the effects persisted in a fifth generation that was not exposed to UV-B treatment, implying that the effects of UV-B irradiation changes could be amplified. This phenomenon could also be apparent in long-lived woody plants.

Damage to DNA caused by UV-B exposure during plant development may not be fully repaired, and thus could be inherited by offspring and accumulated over successive generations in this species (Musil, 1996). Since the genomes of plant reproductive organs are generally well screened from UV-B during both developmental and mature phases (by thick ovary walls and thick anther sacs for male gametes and perianth tissue that has been reported to transmit low UV-B levels), it has been postulated that pollen grains are the most likely candidates for damage by UV-B radiation. Pollen grains have indeed been reported to be particularly susceptible to UV-B radiation during the short period between anther dehiscence and pollen tube penetration into stigmatic tissue (Musil and Wand, 1994; Midgley *et al.*, 1998). Findings of UV-B-induced reductions in pollen viability have also been reported in several South African annual species grown under enhanced UV-B (Musil, 1995). From these findings it has been postulated that pollen grains form an ecologically critical developmental stage of the plant, and in its natural state, *D. sinuata* pollen could be exposed to UV-B during the period between anther dehiscence pollination, and therefore is potentially vulnerable to genetic damage by UV-B. A diminished reproductive capacity with increased UV-B radiation has potentially deleterious consequences for seed bank and population dynamics in natural ecosystems, particularly those of South African semi-arid and arid winter rainfall areas which are subject to periodic natural perturbations as a result of fire and high physicochemical stress (Musil, 1995).

Diminished pollen quality could imply that UV-B radiation interferes with pollen development or that there has been DNA damage in the pollen grains, which could cause mutations during replication of damaged DNA after fertilisation. This would, in turn, alter DNA integrity sufficiently to affect subsequent plant performance (Jiang

and Taylor, 1993). McLennan (1987) has reported the partial purification of the enzyme photolyase from maize pollen and several types of bean, implying that repair of DNA damage to pollen is essential for survival. UV-B irradiation of maize pollen has been reported to activate cryptic transposable elements and under such conditions, recombination processes may also be induced, thus affecting the genome stability of future plant generations (Walbot, 1999).

Even though UV-B has been found to cause changes mainly in somatic tissue that is not going to transmit genes to the next generation, UV-B-induced recombination has been reported in the reproductive (germ) cells, pointing to the likelihood of permanent changes in plant populations as a result of increased UV-B radiation levels (Ries *et al.,* 2000a,b). The progeny of plants exposed to high UV-B levels had a higher somatic mutation rate than their predecessors, suggesting that plants were undergoing heritable and cumulative changes in the expression of genes involved in DNA metabolism. In a recent study, elevated UV-B was found to reduce genome stability in *Arabidopsis thaliana*, and the results suggested that plants were undergoing heritable and cumulative changes in the expression of genes involved in DNA metabolism (Ries *et al.*, 2000a). In the case of *D. sinuata,* if it were not repaired fully, damage to pollen may be inherited by successive generations, and thus accumulate in the genetic material. Seeds from plant material that has been subjected to enhanced UV-B radiation for five generations of the plant are available (Musil 1994, 1995) and were used in this study to address the aims of this study.

1.7 Aims of Study

From the literature it is apparent that there has been a reduction in the amount of ozone in the stratosphere due to man-made CFCs resulting in an increase in UV-B radiation reaching the earth's surface. This increase in UV-B has been found to cause both photomorphogenic as well as genetic changes in plants. Photoreceptors acting through signal transduction pathways are responsible for sensing this ultraviolet radiation. Several components of the photosynthetic apparatus have been found to be affected by UV-B, with nuclear encoded genes being more sensitive to UV-B than chloroplast encoded genes. Despite this plethora of information, long-term effects of UV-B radiation in plants are still not well understood. Studies need to be carried out

over longer time periods to provide definitive answers to questions such as cumulative effects of UV-B, effects of UV-B at ecosystem level, and interactions of elevated UV-B with other stress factors. This study is aimed at elucidating some of these effects and to shed some light on the long-term effects.

The aim of this study is therefore to look at the genetic effects of cumulative exposure to UV-B exposure in an indigenous species *D. sinuata*. Currently not enough is known about the reasons for the large UV-B response differences among treatments/cultivars observed by Musil (see Table 1.0). Ideally it is necessary to understand the genetic basis (heritability) of UV-tolerance and sensitivity. Once this is known, an estimate of the possibility of using conventional breeding practices to minimise the potential effects of UV-damage could be made. At present plant breeders have not yet considered UV-sensitivity as a selective factor.

The changes observed by Musil could possibly be a result of an altered DNA integrity rather than an indirect UV-B effect of photomorphogenic origin, as exemplified by reduced seed size producing smaller seedlings and adult plants. This study is therefore also aimed at establishing whether the observed accumulated effects are genetically based. To achieve this objective, three different approaches were used. In the first, genetic variation in chloroplast DNA between populations of plants from different latitudes were studied. The results of this study could give an indication of the evolutionary history of plants growing in an inherently higher UV-B environment. The second approach employed the use of the *Dra*I assay method of Harlow *et al.* (1994) to look for direct evidence of UV-induced dimerisation in the DNA isolated from study plants. The third approach looked at differences in gene regulation in key photosynthetic pathways using mRNA studies. Biochemical analyses of the same gene products were carried out to correlate mRNA levels with the actual gene products they encode.

CHAPTER 2

EFFECTS OF UV-B RADIATION ON NATURAL POPULATIONS OF THE DESERT ANNUAL *D. SINUATA*: CHLOROPLAST DNA ANALYSIS.

Chapter 2

Effects of UV-B radiation on natural populations of the desert annual *D. sinuata*: chloroplast DNA analysis.

2.1 Introduction

Significant latitudinal variation in incident UV-B radiation has been reported (Green, 1983; Musil *et al.*, 1999b). Solar UV-B increases modestly with elevation above sea level *e.g.* about 14-18% per 1000m elevation. Much larger changes are observed with latitude because of prevailing solar angles. The change of solar UV-B radiation with latitude is large relative to the expected increases of UV-B radiation that would result from ozone reduction. For example, the amount of UV-B experienced at tropical latitudes is greater than in temperate regions because the angle of the sun is such that there is less absorption by the atmosphere. In addition, the natural latitudinal gradient in the ozone layer results in a thin ozone layer in the equatorial regions and a thicker one at the poles (Caldwell *et al.*1989).

A few studies have been carried out in which plant performance across natural solar UV-B gradients and in natural taxa distributed at different elevations was compared. Contrasting sensitivities to UV-B radiation have been reported which implies the presence of natural adaptations to UV-B stress (Caldwell, 1980; Ziska *et al.*, 1992). These indicate that species and ecotypes from high UV-B irradiance environments e.g. montane and low latitude locations, are often less sensitive to elevated UV-B radiation than those from low UV-B irradiance locations with a few exceptions (Ziska *et al.*, 1992; Musil *et al.*, 1999b). This has led to suggestions that genotypic differentiation may have developed among plants along these gradients. Species that are distributed over extensive geographic areas are subjected to large spatial and seasonal variations in solar UV-B exposure (Caldwell, 1980). This is a function of the natural latitudinal gradient in thickness of stratospheric ozone layer, prevailing solar angles at different latitudes, elevation above sea level, cloud amount and form.

Studying UV adaptations in natural plant populations could enable us to find novel or unique protective mechanisms that have not been detected in crop plants already exposed to intensive artificial selection. Perhaps the first place to search for UV-B responsiveness in native plants is in regions where natural levels of UV-B are already

quite high. Plants that naturally occur in high UV environments would undoubtedly have evolved specific adaptations that protect them from the deleterious effects of UV-B. However, this does not mean that they do not respond to UV-B: indeed it might suggest quite the opposite in that their anti-UV mechanisms may be permanently induced. Such plants could also show reduced responsiveness mainly because of reduced sensitivity to UV-B radiation.

Assessing UV-B radiation sensitivity in plants is not easy. This is because sensitivity differs between species and even varieties, and is further influenced by other environmental conditions as well as the developmental history of the plants and geographical origin of the species. It has been hypothesised that species originating from areas that receive high levels of UV-B radiation would be highly resistant to UV-B radiation. There is evidence that species and ecotypes native to low latitudes are inherently more resistant to UV-B irradiation (Caldwell *et al.,* 1989).

Characterisation of chloroplast DNA

Chloroplasts are the chlorophyll-containing, photosynthesising organelle of plants and are thought to be descendants of endosymbiotic cyanobacteria. Each chloroplast is surrounded by a double membrane and contains a system of internal thylakoid membranes, which form stacks of flattened discs called grana in which chlorophyll molecules are embedded. Organelles such as mitochondria and chloroplasts contain their own genomes. Even though chloroplasts are semi-autonomous organelles, their reproduction and functioning is under the control of both nuclear genes and those of the organelle.

The chloroplast genome is the most widely studied plant genome with regard to both molecular organisation and evolution (Clegg *et al.,* 1994). The picture that has emerged from these studies is of a relatively stable genome with marked conservation of gene content and substantial conservation of structural organisation. Chloroplast genomes have a number of prokaryotic as well as eukaryotic features. Even though several chloroplast genes are monocistronic, most chloroplast genes are organised in clusters and are co-transcribed as polycistronic pre-RNAs which are then processed extensively into shorter RNA species. Polycistronic primary transcripts thus obtained

consist of messages for proteins or RNAs with related function, such as in photosynthesis, transcription and translation, and other functions. This indicates that post-transcriptional RNA processing of primary transcripts plays an important role in the control of chloroplast gene expression. Although the chloroplast genomes of many higher plants are maternally inherited (Sager and Lane, 1972; Gillham, 1974; Kuroiwa, 1985; Birky Jr., 1995), most components of the chloroplast genetic system are not coded by the chloroplast but are instead nuclear-encoded. As a result, chloroplasts rely on the nucleus for most of their structural proteins and the regulatory factors that control the expression of their genes. In other words, formation of the chloroplast genetic system requires the co-ordinate expression of nuclear and chloroplast genes (Sugita and Sugiura, 1996).

Vascular plant and algal chloroplast genomes contain approximately 100 genes, most of which encode components of the photosynthetic electron transport machinery and elements of the transcriptional apparatus (Stern *et al.*, 1997). These intracellular organelles contain the entire enzymatic machinery for the process of photosynthesis (Shinozaki *et al.*, 1986a). The chloroplast genomes of higher plants exist as multiple copies of circular, covalently closed dsDNA that range in size from 80-200 kb (Kolodner and Tewari, 1979; Hoffer and Christopher, 1997). The variability in chloroplast DNA size is due to differences in the size of an inverted repeat and small differences in gene content among plant species. A relatively stable arrangement of sequences has been reported in those genomes with a large inverted repeat and a much more dynamic arrangement in those that have lost it (Palmer and Thompson, 1982). It has been postulated that the inverted repeat may play a direct role in maintaining a conserved arrangement of ctDNA sequences. Restriction endonuclease digestion has been used extensively in the past to reveal RFLPs in ctDNA of several plants species (Palmer and Thompson, 1982; Wagner *et al.*, 1987; Chen *et al.*, 1990; Knox and Palmer, 1995, Hultquist *et al.*, 1996). Chen *et al.*, (1990) showed consistently distinguishable restriction endonuclease patterns of ctDNA between fertile and male-sterile cytoplasms of sorghum [*Sorghum bicolor* (L.)].

In general, chloroplast genomes encode a similar group of approximately 100 protein genes, 30 to 31 tRNA genes, and a complete set of rRNA genes, which function in photosynthesis, transcription and translation. Most of these genes code for

components of the plastid's own protein-synthesising apparatus and for many proteins important to photosynthesis i.e., they function either in photosynthesis or as components in the chloroplast protein-synthesising system, (Palmer 1986).

Plants occupy a special niche owing to their ability to obtain energy directly from sunlight for photosynthesis and, as a result, are obliged to be continuously exposed to the ultraviolet (UV) radiation that is present in the spectrum of solar radiation. By virtue of being the light harvesting machinery of the plant, chloroplasts have a significantly greater potential for acquiring ultraviolet induced genetic damage. Like nuclear DNA, organellar DNA is subject to damage (e.g. the induction of pyrimidine dimers) by UV irradiation (Chen *et al.*, 1996). Since the UV component of sunlight is capable of inducing photo-damage in DNA, plants must possess means to prevent DNA damage and, in addition, repair those UV-induced lesions that invariably occur (Cannon *et al.*, 1995). UV-irradiation is probably a major contributor to plastome damage, but since the chloroplast contains DNA that is used as a transcriptional template for gene products essential for photosynthesis and, therefore plant growth and productivity, it is reasonable to assume that a very efficient mode of DNA damage repair is operational in this organelle (Cannon *et al.*, 1995).

Plants sense many aspects of light in their environment, including its wavelength, duration, intensity, and direction. This information is used to optimise growth for the ambient light environment, thereby allowing the plant to function as an efficient photosynthetic machine throughout development (Chory, 1997). Light is essential for normal plant growth and development not only as a source of energy but also as a stimulus that regulates numerous developmental and metabolic processes (Gilmartin *et al.*, 1990). While absorbing visible light energy for photosynthesis, plants are unavoidably exposed to ultraviolet radiation, which is particularly harmful at shorter wavelengths.

Genetic changes induced by environmental stress and the potential impact these changes have on organismal evolution are areas of both great interest and controversy. Stress-induced mutations have been documented in many organisms and are reported to play an important role in the evolution of plants (Waters and Schaal, 1996). Unfortunately, the mechanisms that generate these mutations, the types of stress-

induced mutations that occur in plants and whether or not these mutations are inherited and thus of evolutionary significance are still unknown, except for a few instances. For example, in certain flax varieties, the occurrence of environmentally induced heritable changes has been shown to be accompanied by changes in the genomic DNA (Cullis *et al.,* 1999). Unlike animals, plants do not sequester a germ line early on in development, thus a stress-induced mutation in any cell that later gives rise to reproductive tissue can be passed onto the next generation. Such heritable stress-induced somatic mutations may play a potential role in the evolutionary process.

Findings of UV-B induced reductions in pollen viability in several South African annual species grown under enhanced UV-B have been reported (Musil, 1995). Recently, it has been suggested that, even under experimental treatments using natural white light, damage to the plant genome caused by elevated UV-B may also be inherited by successive generations of the desert annual *D. sinuata* and thus accumulate in the genetic material (Musil, 1996). This form of damage may be extremely important in populations that have rapid turnover of generations, such as annual species, which are common in high-radiation environments. Furthermore, populations which are isolated by habitat fragmentation may be further at risk to this form of damage due to limited outcrossing opportunities.

Based on the observations that plant exposure to episodic or steadily increasing doses of UV-B damages photosynthetic reaction centres, cross-links cellular proteins, and induces mutagenic DNA lesions, it was proposed that *D. sinuata* plants that occur naturally at higher latitudes associated with higher UV-B levels may be physiologically and reproductively less sensitive to UV-B radiation. It was therefore decided that chloroplast DNA (ctDNA) from natural populations would be analysed. Genomic variation in plant populations can be studied using a number of methods. Examples include subtractive hybridization and polymerase chain reaction based methods such as random-amplified polymorphic DNAs (RAPDs) using random arbitrary oligonucleotide primers (Cullis *et al.,* 1999). These methods normally identify DNA changes that are distributed throughout the genome. From these studies, there has been an indication that the susceptible sites depended on the inducing stimulus. Chloroplast DNA restriction endonuclease analysis was used in this study

because T=T sites in the DNA were being proposed as potential candidates for indicating UV-B effects. This is because the chloroplast genome, by virtue of being housed in the light harvesting apparatus, is likely to be targeted by the damaging effects of UV-B radiation. It also has a significantly higher likelihood of acquiring UV-induced genetic damage, especially at T=T sites and this could be tested using restriction endonucleases which target T=T sites in the DNA such as *Dra*I, *Eco*RI and *Hin*dIII.

Growing a selection of *D. sinuata* plants taken from different latitudes (Figure 2.0) in a greenhouse and determining their RFLPs patterns tested this hypothesis. The objective of this chapter was to determine if ctDNA restriction fragment length polymorphisms occur in Namaqualand daisy plants taken from different localities. An analysis of ctDNA was carried out and a comparison of the southern-most latitude plant populations with those growing in an inherently higher UV-B environment (northern latitudes) was conducted. This chapter represents a study of the natural populations of *D. sinuata* that addressed two main questions – firstly whether the natural populations show any evidence of variation in the chloroplast genome, and secondly if the changes could be attributed to prior damage by UV-B *i.e.* via the formation of pyrimidine dimers at some stage in their history. These questions were addressed by analysis of ctDNA using various restriction endonucleases. These included the *Dra*I (TTTAAA), *Eco*RI (GAATTC) and *Hin*dIII (AAGCTT) enzymes whose recognition sequences are possible targets for UV-B radiation, and other enzymes such as *Bam*HI (GGATCC) and *Eco*RV (GATATC), whose recognition sequences are not obvious UV-B targets.

2.2 Materials and methods

2.2.1 Origin of seed material

The seeds that gave rise to the plants used in ctDNA analysis in this study were collected from different regions as shown in Figure 2.0. Even though Aus in Namibia (26°38'S, 16°18'E) is reportedly the northern-most limit for *D. sinuata*, samples were not available from this point, and the northern-most point analysed was Augrabies Falls (28°38'S, 20°25'E), and the southern-most was Kirstenbosch Botanical Gardens,

Cape Town (35°12'S, 18°25'E). Seeds for the Kirstenbosch population were collected from the wild several generations in the past and have since been propagated in the Kirstenbosch Botanical Gardens till the present time. Samples were also collected from Bitterfontein, representing the mid-latitudes (Figure 2.0). MW Van Royen of the University of Pretoria supplied the seeds from Augrabies Falls, (Rosch *et al.*, 1997). Seeds from Bitterfontein were generously supplied by Silverhill Seeds, Kenilworth, Cape Town.

2.2.2 Germination of seed material

Seeds were soaked for five minutes in a 5% solution of sodium hypochlorite, and rinsed five times in distilled water. They were then placed on five layers of moistened Whatman filter paper on petri dishes, which were then sealed with paraffin-wax film to minimise evaporation. Seeds were germinated in the dark for a week before being potted. After six weeks, leaf samples were taken for ctDNA isolation and analysis.

Figure 2.0 Map showing location of sites from which samples were collected.

2.2.3 Isolation and characterisation of chloroplast DNA

All procedures were carried out at 4°C unless otherwise specified. Prior to ctDNA extraction, plants were kept in the dark for 12 hours to destarch the leaves.

Chloroplast DNA was isolated according to the method by Palmer (1986) with minor modifications. Five grams (5 g) of young healthy leaves were harvested and washed

in dH_2O then cut into small pieces. The leaves were homogized in 30 mL of cold isolation buffer (0.35 M Sorbitol, 50 mM Tris-HCl pH 8.0, 25 mM EDTA, 0.1% BSA and 0.1% 2-β-Mercaptoethanol – BSA and 2βME are added just before use) with five 5-second bursts in a milk-shake blender (Sorvall® Model Omni-Mixer 17106, Dupont Instruments). The homogenate was filtered through 4 layers of cheesecloth and then centrifuged at 500 rpm for 5 minutes in a GSA rotor to pellet nuclei and debris. The supernatant was collected and centrifuged at 3000 rpm for 10 minutes at 4°C in a GSA rotor to pellet the chloroplasts. The pellet was then resuspended in 8 mLs of cold wash buffer (0.35 M Sorbitol, 50 mM Tris-HCl pH 8.0, 25 mM EDTA) with the aid of a fine artist's paintbrush. Sucrose gradients (24 mL of 52% and 8 mL 30% sucrose in 25 mM EDTA, 50 mM Tris-HCl, pH 8.0) were prepared and left to stand overnight at 4°C to give a diffuse interface. The resuspended pellet was gently loaded onto the sucrose gradient and centrifuged at 26 000 rpm for 90 minutes in a swinging bucket SW-28II rotor. At the end of centrifugation the chloroplast band was removed with a cut-end blue Gilson pipette tip or a wide-bore pipette and diluted with 5 volumes of wash buffer. This was then centrifuged at 4000 rpm for 15 minutes in an SS-34 rotor and the supernatant was discarded.

2.2.4 Lysis of the chloroplasts

The isolated chloroplasts were lysed either using the lysis buffer method, or they were osmotically shocked in distilled water as described below. With the lysis buffer method, the pellet was resuspended in wash buffer to a final volume of 10 mL and 1/5 volume of lysis buffer [5% (w/v) N-lauryl sarcosinate sodium salt (sarcosinate), 50 mM Tris-HCl pH 8.0, 25 mM EDTA] with 0.1 % (w/v) proteinase K added just before use. The suspension was incubated at 50°C for 15-30 minutes. For the osmotic shock method, the pellet was resuspended in 1 mL of distilled H_2O and transferred to a 2-mL eppendorf tube, and 10 μL of proteinase K (20 mg/ml stock) was added. The resuspended pellet was left at room temperature overnight. The extent of lysis was routinely checked by placing a drop of the lysate on a microscope slide and viewing under a light microscope.

2.2.5 Extraction of nucleic acids

All standard DNA manipulations and electrophoresis were performed as described in Sambrook *et al.* (1989) with some minor modifications. After lysis, chloroplast DNA was extracted at room temperature with an equal volume of TE-buffered phenol, (TE = 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) followed by a phenol: chloroform: iso-amyl alcohol (25: 24: 1) extraction, and finally a chloroform: iso-amyl alcohol (24:1) extraction. The DNA solution (aqueous phase) was transferred to a 30 mL correx tube (for the lysis buffer method), or a 2 mL eppendorf (for the osmotic shock method) containing an equal volume of iso-propanol and 1/10 volume of 10 M ammonium acetate. The DNA was precipitated at room temperature for 30-60 minutes. The DNA pellet was collected by centrifugation, washed in 80% ice-cold ethanol, vacuum dried and then resuspended in 30µl of TE+RNAse A buffer (pH 8.0). DNA concentrations were then determined spectrophotometrically and samples were stored at 4°C.

2.2.6 Restriction endonuclease digestion of ctDNA and agarose gel electrophoresis

The restriction enzymes *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, and *Hin*dIII (Roche Molecular Diagnostics) were used according to the recommendations of the manufacturers. Five microgram aliquots of ctDNA were digested in 30 µl of reaction mixture with 10-20 units of the restriction enzyme and incubated for 12 – 20 hours. Aliquots of the digests were run on a slide gel to check the extent of digestion. The entire digest was then loaded onto a 25 cm-long, 0.8% agarose gel and subjected to electrophoresis for 16 hours at 30V. These samples were used to estimate the ctDNA genome size and to compare the restriction endonuclease digestion patterns of plants from different latitudes.

Estimation of the ctDNA genome size

Equal amounts of chloroplast DNA samples from Kirstenbosch Botanical Gardens were digested with various restriction endonucleases (*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, and *Hin*dIII). Samples were divided into two and were separated by electrophoresis

on 0.6% and 1.5% agarose gels according to Sambrook *et al.* (1989) to resolve the high molecular weight and low molecular weight fragments respectively. Alternatively, digests were resolved by electrophoresis on 0.8% agarose, blotted onto a positively charged nylon membrane according to standard procedures and probed with a DIG-labelled *Bam*HI digest of ctDNA. Five micrograms of ctDNA from Kirstenbosch was digested to completion with *Bam*HI in a 40µl reaction. At the end of digestion, the volume of the reaction mixture was made up to 100 µl with TE buffer. This was then extracted once with phenol/chloroform, and then with chloroform. The supernatant (aqueous phase) was precipitated with an equal volume of isopropanol and 1/10 volume of 5M ammonium acetate. After washing in 70% ethanol, the pellet was resuspended in 15 µl of dH_2O. Five microlitres of this was used to set up a random primed Digoxigenin- (DIG) DNA labelling reaction according to the manufacture's protocol (Roche Diagnostics GmBH, Mannheim, Germany). The labelling reaction was done overnight at 37°C. The concentration of the probe was then determined as described in the supplier's protocol.

To estimate the size of the chloroplast genome, a log plot of the molecular weight marker was made and from this, band sizes were estimated from the distance migrated on a gel. Three enzymes (*Dra*I, *Eco*RI and *Eco*RV) were used in this estimation.

2.3 Results

2.3.1 Estimation of ctDNA genome size

The DIG-labelled ctDNA was used to aid in the detection of modified bands on the blots (Figure 2.3). It was also used to highlight the lower molecular weight fragments that were not easy to discern from the gels. This was crucial in working out the total size of the chloroplast genome. The lysis buffer method resulted in better lysis and this method was used in this study. The restriction patterns of ctDNA from Kirstenbosch, digested with various enzymes are shown in Figure 2.1. This is a representative gel used in the determination of the size of the chloroplast genome of D. sinuata. The chloroplast genome size of D. sinuata was estimated to be 123.80 ± 11.57 kb.

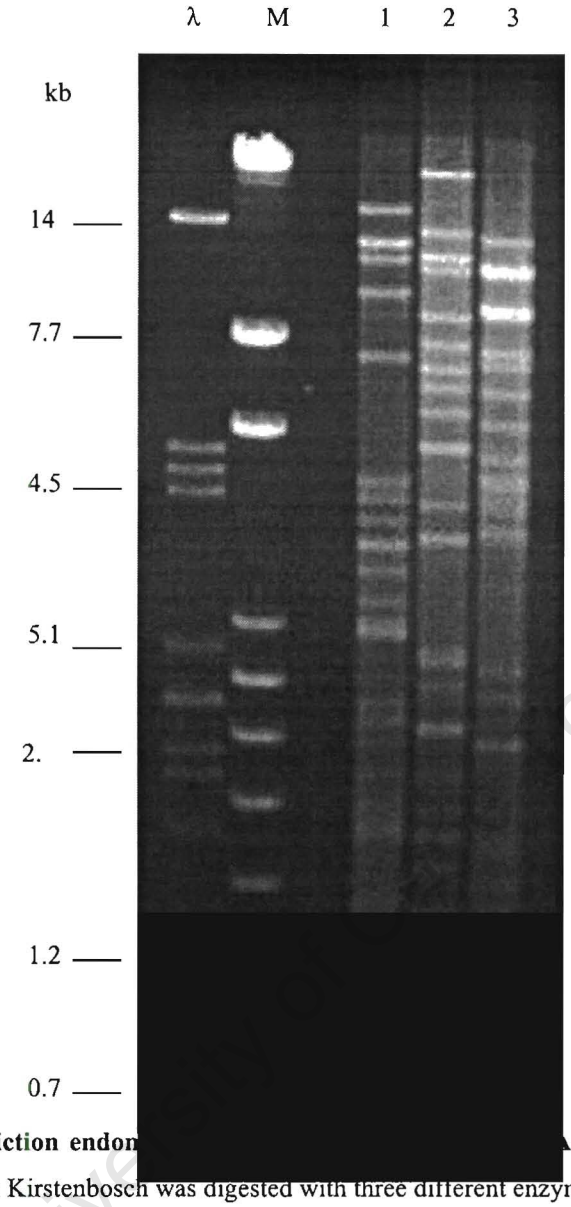

Figure 2.1 Restriction endon **λ.** DNA isolated from *D. sinuata* plants from Kirstenbosch was digested with three different enzymes *Dra*I (lane 1), *Hin*dIII (lane 2) and *Eco*RV (lane 3). Lambda DNA digested with *Pst*I (lane λ) and the high molecular weight marker IV (lane M) (Roche Diagnostics GmBH, Mannheim, Germany) were used as the molecular weight standards.

2.3.2 Restriction endonuclease digestion of ctDNA

Chloroplast DNA from all the sample sites (various latitudes) showed the same pattern when digested with restriction enzymes with the potential dimer formation sites (*Dra*I, *Eco*RI and *Hin*dIII) although the samples were from different sources (Figure 2.2 and 2.3). Analysis of ctDNA from Augrabies Falls samples showed alterations in *Bam*HI and *Eco*RV restriction fragments when compared to those from Kirstenbosch (Figure 2.2). The Kirstenbosch samples were taken as a standard and

were compared to the other sample sites. For the *Bam*HI digest (Figure 2.2 lanes 3 and 4), three polymorphic bands (8.0, 4.8 and 4.0 kb) indicated by arrows, were identified in the Augrabies Falls samples. The 4.8 kb band is not visible on the agarose gel (Figure 2.2) but is more distinct in the Southern blot of the same gel (Figure 2.3, lane 3, indicated by top arrow). This particular band is not very clear in the picture depicted here but it was visible on the actual blot. When the same samples were digested with *Eco*RV (Figure 2.2 lanes 5 and 6), two polymorphic bands of 7.7 and 4.3 kb were identified in the Kirstenbosch sample while the Augrabies Falls sample showed one polymorphic band, 4.5 kb in size (Figure 2.3).

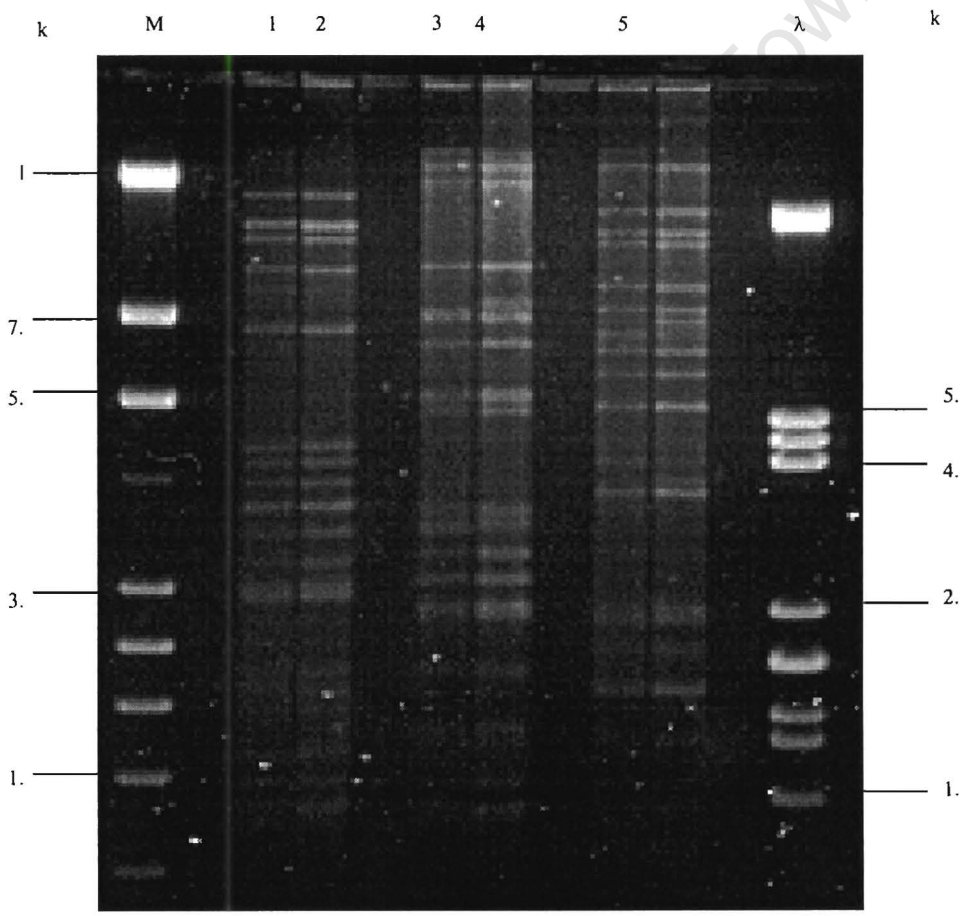

Figure 2.2 Restriction endonclease digestion of chloroplast DNA. Samples from Kirstenbosch (lanes 1, 3 and 5) and Augrabies Falls (lanes 2, 4 and 6) were digested with various enzymes as indicated. Lanes 1 and 2 = *Dra*I; lanes 3 and 4 = *Bam*HI, lanes 5 and 6 = *Eco*RV. M = High molecular weight marker and λ = lambda DNA digested with *Pst*I used as molecular weight standards. Polymorphisms are indicated by the arrowheads.

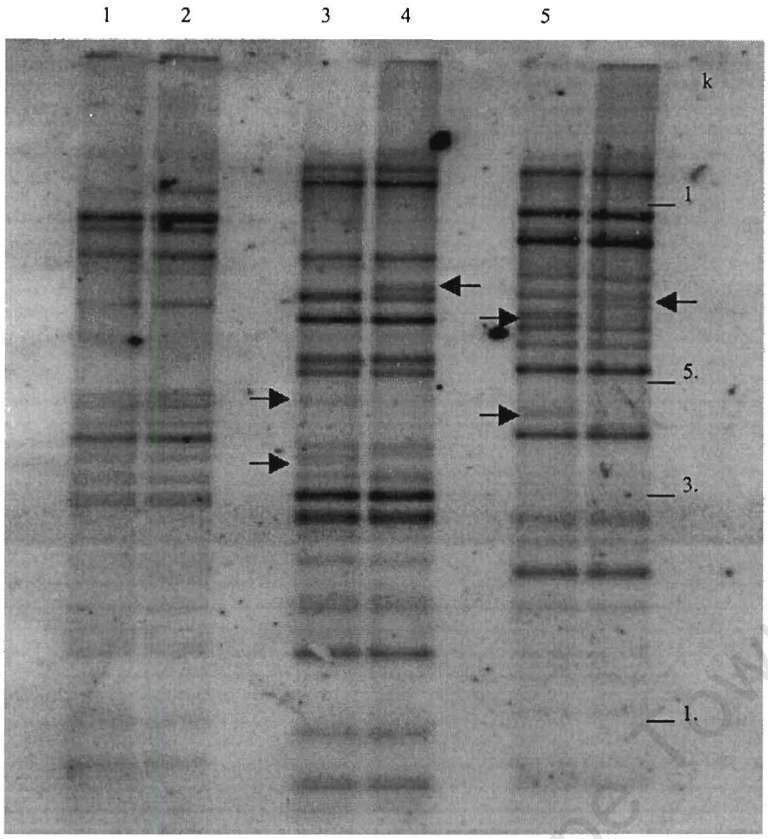

Figure 2.3. Southern blot of chloroplast DNA. Samples from Kirstenbosch (lanes 1, 3 and 5), and Augrabies Falls (lanes 2, 4, and 6) are as shown in Figure 2.1. The gel was probed with DIG-labelled ctDNA that has been digested with *Bam*HI. Lanes 1 and 2 = *Dra*I, lanes 3 and 4 = *Bam*HI, lanes 5 and 6 = E*co*RV. The arrowheads indicate the polymorphic bands from the different restriction endonucleases. Fragment sizes are indicated on the right of the figure.

Figure 2.4 shows chloroplast DNA from different latitudes digested with the *Eco*RV restriction endonclease. The figure is a composite of ctDNA digests of samples from Aurabies Falls, Bitterfontein and Kirstenbosch Botanical Gardens. These samples were resolved on the same gel from which a composite was made for comparison purposes and for clarity. The Bitterfontein samples are very different from either Augrabies Falls or Kirstenbosch.

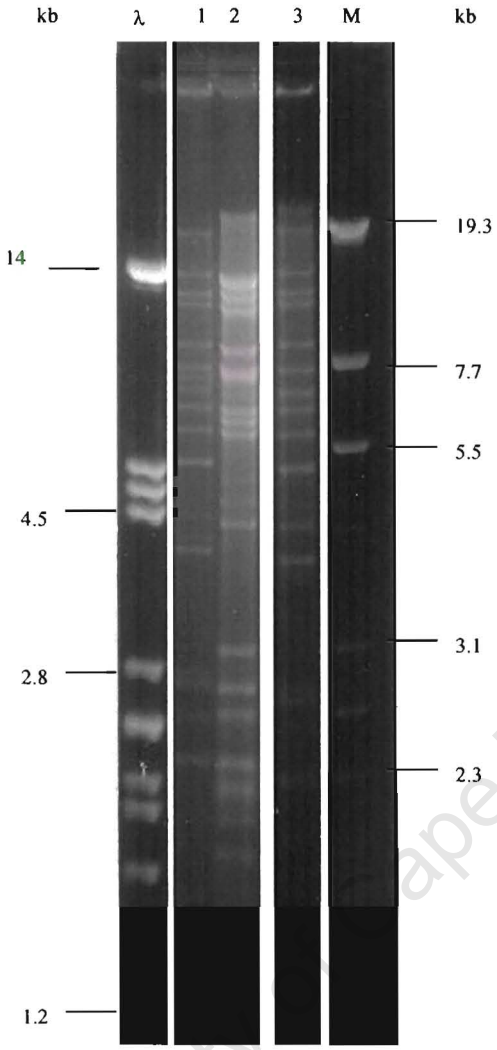

Figure 2.4 Chloroplast DNA restriction endonuclease analysis. CtDNA was digested with *Eco*RV and separated by electrophoresis on a 0.8% agarose gel. Lane 1 = Aurabies Falls, lane 2 = Bitterfontein; lane 3 = Kirstenbosch. λ = λ-*Pst*I molecular weight marker, M = high molecular weight marker IV. Samples were resolved by electrophoresis on the same gel, and a composite is shown here for comparison purposes. See Figure 2.0 for location of sampling sites.

2.5 Discussion

Experimental procedures designed to isolate specific organelles are required to physically separate the organelle from other sub-cellular fractions without disrupting its structural integrity. The cellulose cell walls of most plant tissues are so rigid that the forces needed to disrupt the cell walls tend to rupture fragile organelles as well unless special care is taken during homogenisation. Of particular concern is the large central vacuole, which contains tannins, acids, and hydrolases that can seriously damage the sub-cellular components (Palmer, 1986). Thus the first requirement is

that the cells be disrupted under mild conditions. The second requirement for purification of enzymatically active chloroplasts is that the chloroplasts be protected chemically from the vacuolar contents released during cell disruption. The grinding medium must be sufficiently buffered to stabilise the pH when vacuolar acids are released. In addition, because the chloroplasts are osmotically responsive, the medium must contain an osmoticum similar in concentration to the cytoplasm of the cell, and solutions used for cell fractionation must be kept cold (0-4°C) to arrest activity of hydrolytic enzymes.

A ctDNA isolation protocol was optimised for *D. sinuata* and an analysis of ctDNA profiles of samples from different latitudinal locations was carried out. Two different methods were used to lyse the isolated chloroplasts and the lysis buffer method was found to yield greater quantities of ctDNA compared to the osmotic shock method. The chloroplast genome size of *D. sinuata* estimated to be 123.80±11.57 kb lies within the range reported for chloroplast DNA sizes of higher plants (80-200 kb) (Hoffer and Christopher, 1997).

The chloroplast genome is reportedly highly conserved with respect both to genetic content and an operon-like clustering of certain genes (Clegg *et al.,* 1994; Lidholm and Gustafsson, 1991; Wakasugi *et al.,* 1997). However, an analysis of the entire chloroplast genome of *D. sinuata* has revealed that this is not always the case. An analysis of ctDNA done by comparing ctDNA profiles of plants from Kirstenbosch Botanical Gardens, Cape Town (35°12'S, 18°25'E) to those growing at more northerly latitudes where the plants are exposed to inherently higher UV-B levels showed different restriction patterns. The DNA samples showed alterations in *BamH*I and *EcoR*V restriction fragments and these are enzymes that do not portray obvious UV-B targets in their recognition sequences. On the other hand, those enzymes used in the analysis and selected on the basis of being potential UV-B targets did not show any differences.

This chapter describes the analysis of restriction patterns obtained from chloroplast DNA isolated from natural populations of *D. sinuata* collected from different locations with different UV-B profiles. A number of the polymorphic bands were

found in plants from the different sampling areas experiencing different UV-B regimes but there is no evidence for UV-B causing the changes via mutation at the T=T sites. However, at this stage, one cannot really pinpoint the cause of these RFLPs, whether they are spontaneous or induced mutations. One possibility is that these may be attributed to evolutionary processes acting on this natural population possibly resulting in re-arrangements of the genome, which is in agreement with previous reports that chloroplast genomes usually undergo re-arrangements when subjected to stress (Lidholm and Gustafsson, 1991; Cullis *et al.,* 1999), UV-B radiation being the stress factor in this instance. It is clear from the results that to address this issue, a far more extensive analysis is required but this was clearly beyond the remit of this thesis. The results obtained however, do indicate the potential for this approach and provide some (useful) insight into the complexities involved.

To further enhance the relevance/significance of the results obtained, future studies should look at differences in the sensitivity to UV-B between the samples, and identifying a few plants of different sensitivities and limiting studies to those. From the results, it was concluded that there is a difference between the various samples from the different locations. These may be true but unless the differences in sensitivity are very great, it would always be difficult to elucidate the mechanisms behind the differences based on a limited analysis of chloroplast DNA. Since the differences in sensitivity between the various samples are not known but would have possibly explained the lack of any potential "UV-B-based differences" between the samples, future studies should focus on identifying plant sensitivity to UV-B prior to ctDNA analysis.

Despite a conservative rate of evolution and a relatively stable gene content, comparative molecular analyses reveal complex patterns of mutational change (Clegg *et al.,* 1994). Non-coding regions of ctDNA are reported to diverge through insertion/deletion changes that are sometimes site dependent. The observed differences clearly indicate the existence of genetic variation in the chloroplast genome of the Namaqualand daisy. This suggests that there might be some geographically significant genetic structure in *D. sinuata*. This could be characterised further by looking at more samples from different populations. It was postulated that

samples from northerly latitudes are likely to have experienced continued UV-B exposure, but there was no obvious distinction in genetic structure with latitude. For example, restriction endonuclease digestion of ctDNA from Bitterfontein and Augrabies Falls which are at more northerly latitudes showed different patterns to Kirstenbosch plants. Knowledge of the plants' UV-B sensitivity would also shed light on the genetic structure of the different populations and the likely existence of heterogeneity in plants from the different populations, and the existence of distinct geographic patterning of populations. Future studies could also focus on determining the level of polymorphism between ecotypes of other species growing in the same environment as this would then shed some light on the sensitivity of these particular plants to UV-B. The samples from Bitterfontein showed very different patterns from either the Augrabies Falls or Kirstenbosch Botanical Gardens sites, clearly indicating the existence of genetic variation and different population structures at both sites.

Even though polymorphisms exist in samples from all three sites, there is less variability between the Augrabies Falls (a more northerly latitude) and the Kirstensbosch samples than there is between Bitterfontein samples (mid latitude) and Kirstenbosch Botanical Gardens. This rules out the variability in UV-B with latitude as being the cause of the observed differences. Since none of the observed genetic variation could be attributed to direct UV-B effects, there is a high possibilty that the observed differences may be due to a number of stress factors that play a pivotal role in the evolutionary history of the Namaqualand diasies in their natural environment. Extensive occurence of environmentally induced heritable changes in plants has been reported in a recent paper (Cullis *et al.,* 1999).

Another possible mechanism of genetic modification is via transposition. UV-B has been reported to activate transposons, especially the immobile Mutator (*Mu*) transposons in maize sperm (Walbot, 1999). These transposons amplify the effects of UV-B exposure by causing mutations beyond the extent of immediate DNA damage. Increasing terrestrial UV-B amounts have been reported to be cytotoxic to pollen (since haploid pollen has been reported to be a sensitive target of UV-B irradiation), thereby increasing the frequency of new mutations. Activation of cryptic transposable elements could also increase the mutation rate. Such transposons can be activated by genomic shock as an adaptive mechanism. Whereas damage to DNA is immediately

repaired or fixed as stable mutations, transposons produce cycles of insertion and excision long after activation. Therefore large genomes may be particularly susceptible to destabilisation. For instance, about half of the maize genome is constituted by inactive retrotransposons, serving as a reservoir of potential mutagens (Walbot, 1999). Because plants lack a dedicated germ line, mutations in somatic stem cells can be represented in the subsequent gametes. Normally, stringent selection on the vegetative cell of haploid pollen maturation and growth counterbalances the accumulation of deleterious alleles in the diploid soma. Mutations induced in the individual sperm are not subject to such rigorous selection, because genetic activity is thought to start after fertilisation. As a result, transposon activation in individual sperm could greatly increase the genetic load in higher plants.

Ericaceae (perennial shrubs < 1m in height) constitute an important component of the natural vegetation of the South African Mediterranean climate zone. Some of these plants occur in strongly leached, nutrient-impoverished soils in semi-arid areas of the southern and south-western Cape (Bond and Goldblatt, 1984; Musil and Wand, 1993). In such environments, plant growth rates are generally low, and excess carbon is partitioned with secondary phenolic compounds, such as flavonoids, which have a substantial UV-B absorbance and potential antioxidant properties, yet are largely transparent in the visible portion of the solar spectrum. Since the Asteraceae occur in similar environments as the Ericaeae, there is a possibility that such stresses together with UV-B could be contributing factors to the genetic variation observed in natural populations of the Namaqualand daisy.

Contrasting sensitivities to UV-B radiation have been reported in natural taxa distributed at different elevations, which implies the presence of natural adaptations to UV-B stress. To understand the process of chloroplast genome evolution, information on repeated sequences, intergenic regions and pseudogenes in chloroplast DNA is extremely useful (Wakasugi *et al.,* 1997). There can be no doubt that the primary response to UV radiation is shielding via pigment and wax production and associated cuticular thickness. To address these questions, the next step was to carry out pigment analysis in plants grown under controlled conditions simulating a predicted stratospheric ozone reduction of 20% (Chapter 3).

CHAPTER 3

PHYSIOLOGICAL AND BIOCHEMICAL STUDIES ON THE CUMULATIVE EFFECTS OF ELEVATED UV-B ON PHOTOSYNTHETIC PIGMENTS OVER MULTIPLE GENERATIONS OF *DIMORPHOTHECA SINUATA*

Chapter 3

Physiological and biochemical studies on the cumulative effects of elevated UV-B on photosynthetic pigments over multiple generations of *Dimorphotheca sinuata*.

3.1. Introduction

3.1.1. Photosynthesis

Photosynthesis is one of the most important biological processes on earth. Because of this, scientific research into photosynthesis is extremely important. If we can understand the intricacies of the photosynthetic process, we can learn how to increase crop yields, fiber, wood, and fuel, and how to use our lands in a more benign way. The energy-harvesting secrets of plants can be adapted to synthetic systems which will provide new, efficient ways to collect and use solar energy. Because photosynthesis contributes to the makeup of our atmosphere, understanding this process is crucial to understanding how carbon dioxide and other greenhouse gases affect the global climate.

During photosynthesis, the energy of the absorbed light is converted into chemical and electrochemical energy which is used to support cell growth. The process occurs in pigment-protein complexes called reaction centers, which are embedded in thylakoid membranes (Raven and Johnson, 1990; Anderson and Beardall, 1991). There are two photosystems that work sequentially during the process. First, when a photon of light strikes a pigment molecule in photosystem II (PS II), it excites an electron and this high energy-electron is ejected from PS II. This electron is coupled to a proton stripped from water by a Z protein and is passed along a chain of membrane-bound cytochrome electron carriers to a proton pump. There the energy supplied by the photon is used to transport a photon across the membrane into the lumen of the thylakoid. The resulting proton gradient drives the chemiosmotic production of a molecule of ATP. The electron then passes to photosytem I (PS I) along a chain of cytochromes. Secondly, when PS I absorbs another photon of light,

its pigment passes a second high-energy electron to a reduction complex, which drives NADPH generation (Anderson and Beardall, 1991).

Research into photosynthesis is at a particularly exciting stage since the molecular structures of many of the relevant proteins and pigment-protein complexes are now being characterized in detail (Lichtenthaler, 1987; Anderson and Beardall, 1991; Barber and Anderson, 1994; Baker *et al.,* 1997). The main focus of photosynthesis research has been the functional and structural aspects behind the energy transducing process. The progress has been significant, and the photosynthetic protein complexes can now be described at a very refined molecular level. Amino acid sequences of important polypeptides are becoming available, as is 3-dimensional structural information from X-ray diffraction and electron microscopy. Model systems are being constructed to help elucidate the chemistry and physics of the *in vivo* system, and to discover principles for possible application in the construction of efficient solar cells. However, relatively little is known about the acclimating, regulatory, and protective processes that maintain high photosynthetic efficiency during ever fluctuating and even stressful environmental conditions (Yang *et al.,* 1998). The identification of such auxillary processes associated with thylakoid membranes and the characterisation of the enzymes involved are therefore central tasks of current photosythesis research.

3.1.2 Photosynthetic pigments

The photosynthetic pigments chlorophylls and carotenoids, together with sterols, prenylquinones, and prenols, belong to the group of isoprenoid plant lipids, the so-called prenyl lipids (Lichtenthaler, 1987). Carotenoids as tetrapenoids are simple or pure prenyl lipids, the carbon skeleton of which is made up solely of isoprenoid units. Chlorophyll *a* and *b* are mixed prenyl lipids and they posses an isoprenoid chain which is bound to a non-isoprenoid porphyrin ring system.

The chlorophylls of higher plants consist of chlorophyll *a* as the major pigment and chlorophyll *b* as an accessory pigment. Both chlorophylls are genuine components of the photosynthetic membranes and occur in a ratio (chlorophyll *a/b*) of approximately 3:1. Growth conditions and environmental factors can modify the chlorophyll *a/b*

ratio (Lichtenthaler, 1987). For example, high light and sun-exposed plants (high-light chloroplasts) exhibit chlorophyll *a/b* ratios of 3.2 to 4, whereas shade plants (low light chloroplasts) possess lower ratios of 2.5 to 2.9.

The group of primary plant carotenoids can be divided into the oxygen-free carotenes and into the xanthophylls which contain oxygen in different forms. The xanthophylls comprise a group of oxygenated carotenoids with structural and functional roles in the photosynthetic antennae complexes of higher plants and algae. They can function as accessory light-harvesting pigments, structural entities within the light harvesting complex (LHC) and as molecules required in photoprotection of photosynthetic organisms from the potentially damaging effects of light (Masojidek *et al.,* 1999). Since xanthophylls contain oxygen in different forms (such as one or several hydroxy groups), two types of isomers exist, one on each oxidation level, the α-carotene derivatives and the β-carotene derivatives. Introduction of hydroxy and epoxy functions into α-carotene gives rise to lutein and lutein epoxide. β-carotene is the precursor of the xanthophylls zeaxanthin, violaxanthin and antheraxanthin. The carotenoids of green, photosynthetically active plants, which are needed for photosynthetic function, are classified as primary carotenoids, whereas those of red fruits and flowers have been termed secondary carotenoids.

The carotenoids of functional chloroplasts include β-carotene, lutein, violaxanthin, and neoxanthin as major regular components of the photochemically active thylakoids of higher plants and green algae. β-carotene may partially serve as a light-absorbing pigment: its main function, however, seems to be the protection of chlorophyll *a* from photooxidation in or near the reaction centre (Lichtenthaler, 1987). Except for violaxanthin, which to some extent is bound to the chloroplast envelope, all carotenoids and chlorophylls are bound to the thylakoids, the photochemically active photosynthetic biomembranes. Within the thylakoids, the pigments are associated with several chlorophyll-carotenoid proteins that possess differential chlorophyll composition. For example, the major light-harvesting complex of higher plants is the most common pigment-protein complex on earth constituting more than 40% of the photosynthetic membrane protein. Oxygenic photosynthesis is largely reliant upon

the efficiency and adaptability of this complex to collect light energy and deliver it to the reaction centres of photosynthesis (Ruban *et al.*, 2000).

Since these pigments have been reported to be modified by growth conditions and environmental factors, and because UV-B also has several effects on the physiology of terrestrial plants, it was felt that a determination of levels of these pigments would help elucidate the effects of UV-B on the physiology of *D. sinuata*. With enhanced UV-B some of the induced changes which can modify subsequent plant responses include anatomical alterations, quantitative and qualitative changes in epicuticular wax, and pigment changes among others. Potential biochemical changes include the xanthophyll cycle. This cycle is a protective mechanism for the dissipation of excess amounts of visible radiation, otherwise known as photoinhibition. The degree of damage caused by UV-B should be strongly dependent on the efficiency of constitutive and UV-induced mechanisms of protection and repair, such as the accumulation of UV-absorbing sunscreens and the activation of antioxidant defences (Mazza *et al.*, 2000).

It should be noted however, that UV-B radiation exerts opposing effects on plants. Because it is of high energy, UV-B radiation causes damage to DNA and protein, lipid peroxidation, and pigment oxidation (Kim *et al.*, 1998). On the other hand, UV-B can provoke plant photomorphogenic responses, some of which appear to be adaptive. These include hypocotyl growth inhibition, cotyledon curling, and rapid induction of expression of a variety of genes, including those involved in synthesis of phenylpropanoid and flavonoid UV sunscreens (see chapter 1).

In the earlier stages of this study, accumulated UV-B was found to have a greater effect on plant performance than immediate effects (Musil 1996). The former could be identified by earlier reproductive effort, substantial (up to 35%) reductions in dry mass (stems, leaves and reproductive organs), decreased stem and inflorescence production, diminished steady-state flourescence yields, chlorophyll *a* concentrations, pollen tube growth and germination of seeds set. The latter caused only diminished non-photochemical quenching, reduced chlrophyll *a* concentrations, reduced soluble sugar and starch concentrations, decreased pollen germination and increased carotenoid contents. With the above in mind, an experimental design was embarked

upon which addressed whether previously observed attributes in the test plants (Musil, 1996) were of a photomorphogenic nature or had a genetic basis.

The aim of this section of the study was therefore, to look at both photosynthetic pigment and secondary compound levels in two groups of plants: those with a history of enhanced UV-B exposure and those with a history of ambient UV-B.

3.2 Materials and methods

3.2.1 Cumulative UV-B treatments

Populations of the desert annual *D. sinuata*, derived from a common seed stock (obtained from populations in the National Botanical Gardens, Kirstenbosch, Cape Town, South Africa), were exposed concurrently over four successive generations to either ambient (representing no stratospheric ozone depletion), or elevated (representing 20% stratospheric ozone depletion) UV-B levels during the complete life cycles (Figure 3.1). The ambient UV-B group was exposed to UV-B fluences approximating those received daily over the natural growing period of *D. sinuata* at its southerly distribution limit (33°56'S, 18°29'E: Cape Town, South Africa) (a seasonal range of 2.55-8.85 kJ/m^2/day), while the enhanced UV-B group was exposed to UV-B fluences simulating those at the northerly distribution limit of this species (26°38'S, 16°18'E: Aus, Namibia) (a seasonal range of 4.70-11.41 kJ/m^2/day) (Musil, 1996). The UV treatments lasted over the full species' growth cycle and were given over the natural growing period (mid winter or late spring).

Seeds were sown at a depth of 10 mm in potting medium comprising coarse sand, leaf mould and loam (2:1:1, v:v) contained in 20 cm diameter pots. UV-B treatments commenced when seeds started germinating (Musil, 1995). Pots were irrigated daily with equivalent volumes of water and fertilised at 2-weekly intervals with 5.8 mg nitrogen, 0.8 mg phosphorus and 1.7 mg potassium per kg of dry soil. The first two generations were grown in the absence of natural UV-radiation in a polycarbonate-cladded greenhouse (with no transmission below 400 nm) where UV-B radiation at ambient and enhanced levels was supplied exclusively from artificial sources. Peak daily photosynthetic photon flux densities (PFD) in the greenhouse ranged seasonally

(spring to midsummer) from 600 to 1800 μmol/m^2/s (Musil, 1996). Lamps above treatment plants were filtered with 0.075 mm thick cellulose acetate film (Coutaulds Chemicals, Derby, UK) with transmission down to 290 nm (which was replaced weekly). For control plants receiving ambient UV-B levels, lamps were filtered with 0.12 mm thick Mylar –D film (DuPont De Nemours, Wilmington, Delaware, USA) with no transmission below 316 nm. This was done in accordance with reports on the importance of UV-A radiation and total photon flux ratios of UV-B:UV-A and UV-B:PFD as mitigating factors in plant responses to UV-B (Middleton and Teramura, 1993; Caldwell *et al.*, 1994). In the third and fourth generations, these population groups were exposed to either ambient or ambient plus elevated levels of UV-B outdoors in an open natural setting, simulating approximate field growth conditions. In the fifth generation, random seed samples of both treatments from generation four were grown in the greenhouse in the absence of further UV-B radiation exposure. Dr Charles Musil of the National Botanical Institute, Kirstenbosch, Cape Town carried out the initial stages of the study and then generously supplied seed material from generation four plants, which were used in the study.

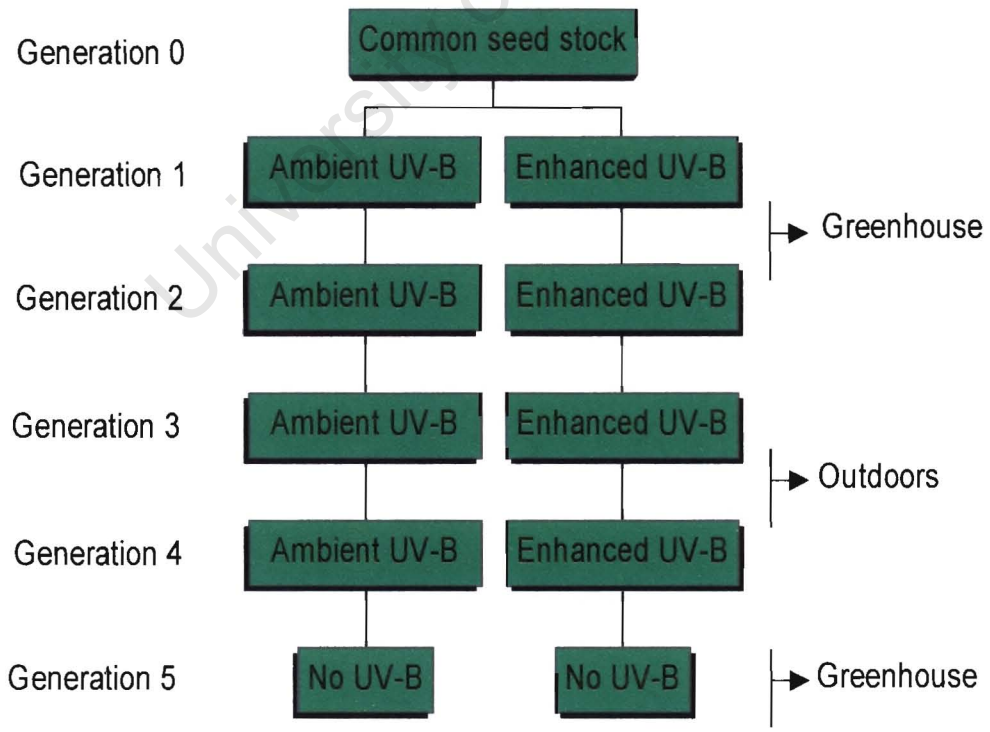

Figure 3.1 Schematic representation of experimental design showing sequence of treatments and derivation of experimental material used in the study.

3.2.2 Germination of seed material and growth of plants in the absence of UV-B treatment.

Seeds from the fourth generation were soaked for five minutes in a 5% solution of sodium hypochlorite, and rinsed five times in distilled water. The seeds were then placed on five layers of moistened Whatman filter paper on petri dishes and these were sealed with paraffin-wax film to minimise evaporation. Seeds were germinated in the dark for three days before being potted and were watered daily thereafter. The standard conditions in the growth room were as follows: temperature = 22°C, relative humidity = 65%, 16 hours light and 8 hours darkness with a light intensity of ± 100 μmol/m^2/s. After six weeks, leaf samples were taken for DNA and RNA isolation, and for biochemical analysis. These plants were designated generation five (Figure 3.1).

3.2.3 Extraction of pigments and metabolites

Photosynthetic pigments and secondary compounds were determined from either fresh leaves or liquid-nitrogen frozen leaf material sampled from six-week old plants from the fifth generation offspring. Four mature leaves were randomly selected from mid-axial positions on each plant. The leaves were subdivided into manageable samples of approximately equal size and placed in plastic 15 ml containers that could withstand freezing and thawing. The leaves were immediately used for pigment analysis or flash-frozen in liquid nitrogen and then stored at –70°C.

In each subdivided leaf sample, photosynthetic pigments were extracted in 10 ml of 100% methanol at 2°C in the dark. Absorbencies of filtrates were measured with a spectrophotometer (Beckman DU640, Beckman Instruments Inc., Fullerton, USA) at specified wavelengths required for computation of chlorophyll *a* (665.2 nm), chlorophyll *b* (652.4 nm) and total carotenoid (470.0 nm) concentrations (Lichtenthaler, 1987). Precipitates were dried at 60°C in a forced draft oven and weighed. Concentrations were expressed per unit dry mass.

Secondary compounds (total polyphenolics and anthocyanins) were extracted in 10 ml of acidified methanol (79:20:1), v:v, methanol:water:HCl). Absorbances of filtrates were measured at 657 nm and 530 nm, and at 300 nm after appropriate dilution. Precipitates were dried at 60°C in a forced draft oven and weighed. Total phenolics (methanol-extractable UV-B absorbing compounds) were computed from measured absorbances (A_b) at 300 nm (Mirecki and Teramura, 1984). Anthocyanins were computed from A_b530 nm – 1/3 A_b657 nm (Lindoo and Caldwell, 1978). Absorbance values were expressed per unit dry mass. For each sample, readings were taken in duplicate and the overall means computed.

Statistical analysis

A single-factor nested analysis of variance (subdivided leaf samples comprising nested measures within each plant sample) was used to test for significant differences in foliar pigment and secondary metabolite concentrations between offspring from the two differently UV-B irradiated ancestral populations (enhanced UV-B treatments and the ambient UV-B controls).

3.3 Results

Plants grown from seeds from the enhanced UV-B treatments showed significant reductions in foliar chlorophyll *a*, total carotenoids and total photosynthetic pigments and an increase in secondary metabolites as a function of dry mass (Table 3.1). Statistical analysis showed that these reductions were significant at the 5% level of significance. Chlorophyll *b* levels also showed reductions in the progeny from an enhanced UV-B level history but these were not statistically significant at the 5% level of significance. Of the increased levels of secondary metabolites in the enhanced UV-B group, only anthocyanins showed significant differences at the 5% level of significance. The increase in phenolics did not attain statistical significance (Table 3.1).

Table 3.1. Statistical analysis of the photosynthetic pigment and secondary metabolite concentrations in leaves of *D. sinuata* offspring expressed per unit leaf dry mass.

Parameter	Ancestors from ambient UV-B environment	Ancestors from elevated UV-B environment	Analysis of Variance	Significance level [b]
Chlorophyll *a* μg mg^{-1}	**21.88**[a]	**19.18**	$\mathbf{F_{1,45} = 27.73}$	***P ≤ 0.001***
Chlorophyll *b* μg mg^{-1}	6.72	6.46	$F_{1,45} = 2.12$	P ≤ 0.142
Chlorophyll *a/b ratio*	**3.44**	**3.02**	$\mathbf{F_{1,45} = 16.53}$	**P ≤ 0.001**
Carotenoids μg mg^{-1}	**2.97**	**2.65**	$\mathbf{F_{1,45} = 15.27}$	**P ≤ 0.001**
Total chlorophylls μg mg^{-1}	**28.60**	**25.64**	$\mathbf{F_{1,45} = 18.74}$	**P ≤ 0.001**
Total photosynthetic pigments μg mg^{-1}	**31.57**	**28.29**	$\mathbf{F_{1,45} = 19.39}$	**P ≤ 0.001**
Phenolics Abs (300 nm) g^{-1}	94.10	98.30	$F_{1,45} = 3.69$	P ≤ 0.056
Anthocyanins Abs (530 nm) g^{-1}	**7.29**	**7.96**	$\mathbf{F_{1,44} = 4.55}$	**P ≤ 0.034**

[a]Each value represents a mean of at least three independent observations of twenty-one plants from the ambient UV-B group, and twenty-six plants from the elevated UV-B group. [b]Significant ($P \leq 0.05$) contrasts presented in **bold** type.

Foliar pigment and metabolite concentrations in fifth generation *D. sinuata* offspring are represented graphically. The graphs are a scatter-plot showing the distribution of pigment and metabolite concentrations, with each point representing a mean of at least 4 observations for a given plant. Photosynthetic pigments (which showed a decreased trend in the elevated UV-B group) are shown in Figure 3.2 graphs a to c, and secondary metabolites which were found to increase in the elevated UV-B group are depicted in Figure 3.3 graphs a and b.

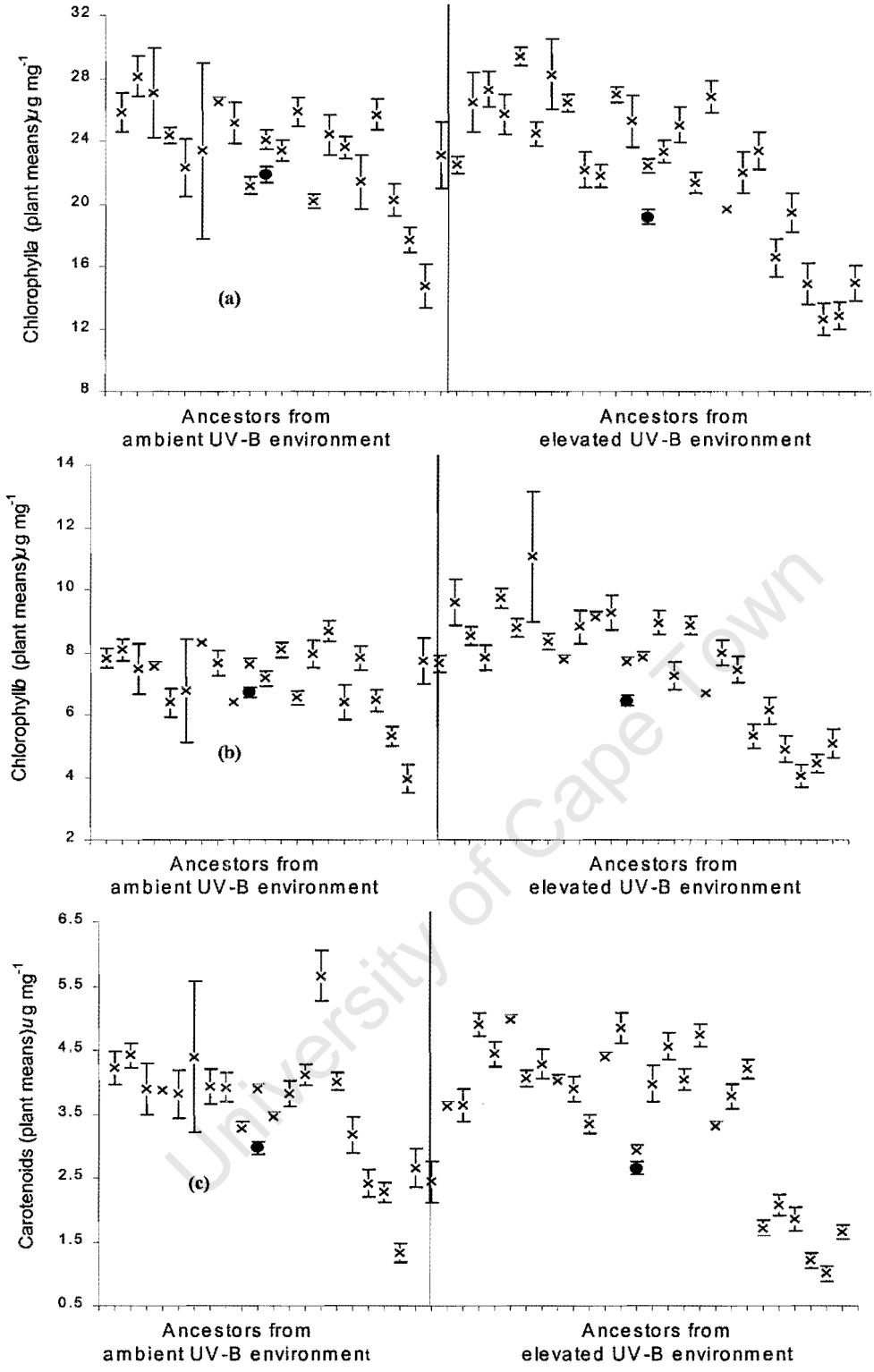

Figure 3.2. Foliar pigment and metabolite concentrations in progeny of *D. sinuata* derived from ancestors with either an ambient- or an elevated-UV-B history. Photosynthetic pigments are shown in graphs a to c : (a) chlorophyll *a* levels; (b) chlorophyll *b*; (c) carotenoids. Values are shown as means of at least four observations (vertical bars represent two standard errors). x =individual plant means of at least three observations, ● = overall mean for each group, I =standard error.

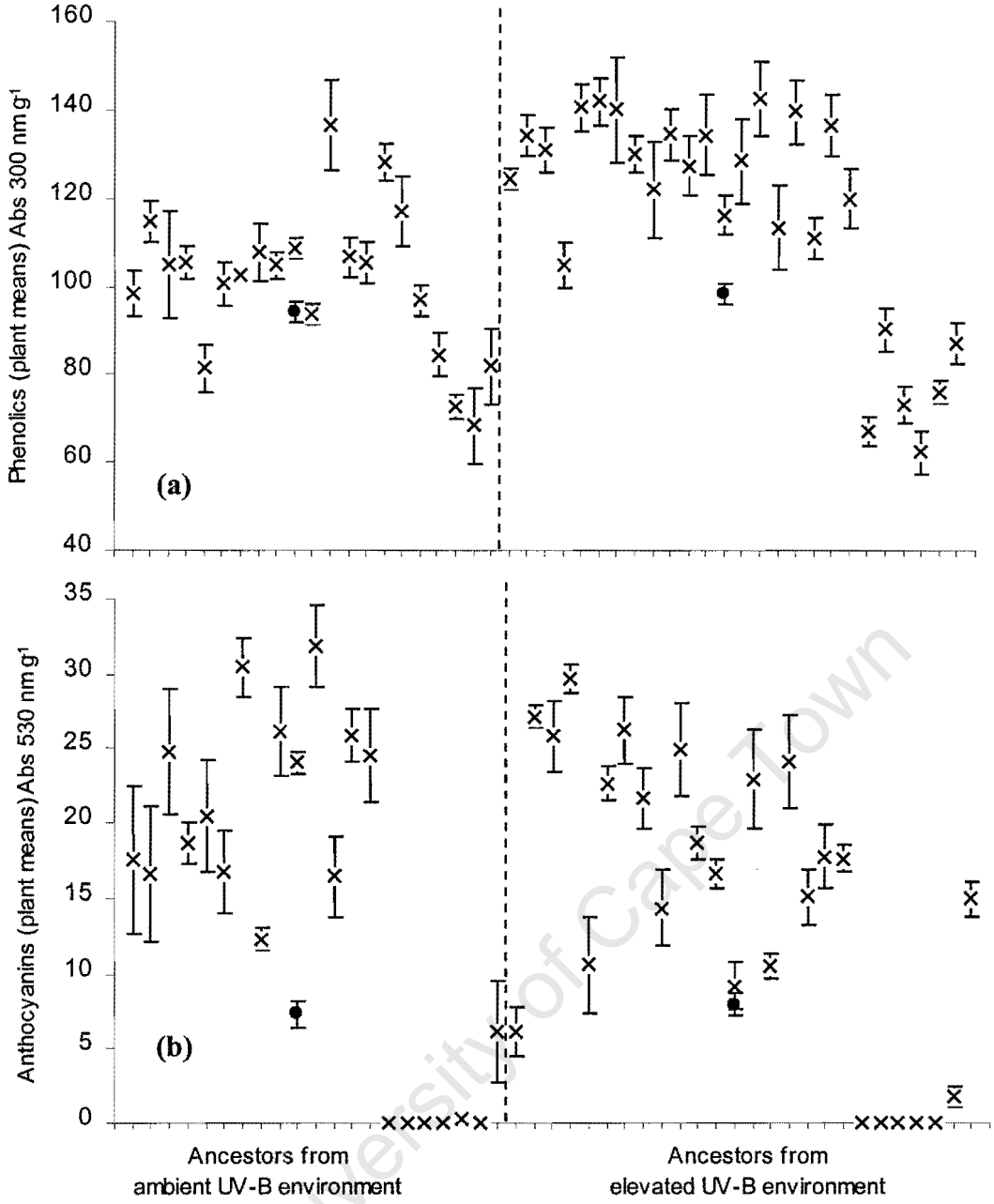

Figure 3.3. Foliar pigment and metabolite concentrations in progeny of *D. sinuata* derived from ancestors with either an ambient- or an elevated-UV-B history. Secondary metabolites are shown in graphs a and b: (a) phenolics; (b) anthocyanins. Values are shown as means of at least four observations (vertical bars represent two standard errors). x =individual plant means of at least three observations, ● = overall mean for each group, I =standard error.

3.4 Discussion

Of the measured leaf parameters from the progeny of plants with a history of elevated UV-B exposure, only chlorophyll *b* and phenolics did not show significant differences at the 5% level of significance. A reduction in all the photosynthetic pigments

measured was observed (Figure 3.2, a to c) and an increase in secondary metabolite levels (Figure 3.3, a and b). This suggests that such parameters may be heritably affected by repeated exposure to enhanced UV-B radiation. Even though an increase in total phenolics was observed in the elevated UV-B group, it did not attain statistical significance (Table 3.1 and Figure 3.3 a). For all the measured parameters, the last six observations in the graphs represent values obtained from frozen material. These values are generally lower than the rest of the values and could be the result of the effects of freezing and storage on the pigments, especially the anthocyanins which are water solube and very labile. The specific leaf area and specific leaf mass were determined for this group of plants. There was a tendency towards thinner leaf area in higher UV-B plants. Statistical tests were done on pigment and metabolite concentrations expressed either as a function of dry mass or per unit area. There was no difference in the level of significance obtained for both parameters i.e. morphology did not have any compounding effect on pigment and metabolite concentrations. The observed differences were, therefore, probably due to significant change in photosynthesis and productivity. In addition, photosynthetic rates were measured in plants in another study taken from the same seed set and similar results were found (Musil *et al.*, 1999a).

In the early stages of this study in which seeds were taken from the same common stock but grown under ambient and enhanced UV-B conditions (generations one to four), an enhanced UV-B history was found to have greater negative effects on gross plant characteristics such as total leaf area, leaf dry mass, and stem mass (Musil, 1996). Foliar chlorophyll *a* concentrations, steady-state fluorescent yields, pollen tube growth rates and the germination success of seeds set were all significantly lower in the enhanced UV-B group (Musil, 1996). Seeds from the plants from these earlier studies were used in this section of the study but in the absence of further UV-B exposure. The results from generation 5 confirm the hypothesis that the modifications in the plants resulting from prolonged UV-B are heritable as they were observed in the absence of UV-B radiation. This could be an indication that gene regulation has been modified in these plants during past exposure to UV-B radiation.

Results from previous studies (Midgley *et al.*, 1998; Musil, 1996; Musil *et al.*, 1999a), in combination with the present study in which plants were grown in an essentially

UV-B-free environment, point to the heritability of several photosynthetic parameters. Important targets of UV-B radiation include nucleic acids, proteins and membrane lipids. Damage to these key elements will reflect a direct alteration in a number of key photosynthetic reactions (Teramura *et al.*, 1996). Together, the above constitute good evidence that some aspect of gene regulation could have occurred during exposure of the parent material to enhanced UV-B conditions. In addition, facets of the photosynthetic apparatus, or the regulation of genes involved in photosynthesis, could have been affected by this history of enhanced UV-B exposure.

Of special note is the non-significant reduction in the level of chlorophyll *b* observed in the elevated UV-B group. Musil *et al.*, (1999a) reported an observed trend of increasing foliar chlorophyll *b* levels with cumulative generations of enhanced UV-B exposure. However, this was most pronounced when the offspring were grown in the presence of other stresses such as limiting nutrients and CO_2 levels. This contrasting observation could be explained by the fact that in this study the plants were grown under normal greenhouse conditions in the absence of any stress. It is possible that more carbon was allocated to the protection mechanisms in the epidermis (secondary wall growth, and the synthesis of wall-bound phenolics) at the expense of photosynthetic area. A reduction in productivity of plant irradiated with UV-B throughout development is usually associated with a reduced ability to intercept light due to a smaller leaf area and not an inhibition of photosynthetic competence (Allen *et al.*, 1998). The subtle changes in leaf area reported previously (Musil, 1996) could lead to reductions in the whole plant photosynthetic area and probably total plant carbon assimilation, which has been suggested as an explanation for the possible accumulation of growth reduction in response to UV-B (Laakso *et al.*, 2000; Sullivan and Teramura, 1992; Sullivan, 1994; Sullivan *et al.*, 1994).

It is, therefore, conceivable that the significant differences observed in the enhanced UV-B group may have been initiated by altered molecular signals resulting from DNA damage. The diminished chlorophyll *a* and *b* concentrations measured in the enhanced UV-B group could imply modification in the regulation of genes involved in photosynthesis, since down-regulation of these genes by UV-B radiation has been reported to cause substantial losses in chlorophyll-protein content and activity that led to diminished photosynthetic capacity (Strid *et al.*, 1994).

Since the results from these experiments and those reported previously in the earlier stages of the study (generations one to four) (Musil, 1996) point to the likelihood of heritable and cummulative DNA damage by UV-B, experiments were designed to assay for evidence of UV-B induced mutagenesis. This included the *Dra*I assay discussed in Chapter 4, and the cloning of the *rbc*L gene which was used as a probe to look at variation in the study plants both at DNA and mRNA levels (see Chapters 4 and 5).

CHAPTER 4

CLONING/CHARACTERISATION OF THE *RBC*L GENE AND THE *DRA*I ASSAY FOR INVESTIGATING EVIDENCE FOR UV-B INDUCED MUTAGENESIS

TABLE OF CONTENTS

Chapter 4

Cloning/characterisation of the *rbc*L gene and the *Dra*I assay for investigating evidence for UV-B induced mutagenesis

4.1 Introduction

DNA is a highly reactive molecule that is sensitive to damage from a wide range of both physical and chemical agents. Nuclear DNA is present in very low copy number and acts as a template for its own synthesis. Because of this, it is a vulnerable target for UV-induced damage. Lesions alter the structure of DNA and consequently interfere with critical aspects of DNA metabolism such as transcription, replication, and recombination. Even a single persisting UV-induced lesion can be a potentially lethal event, particularly in haploid tissue such as pollen grains (Britt, 1997).

The maintenance of genetic integrity is essential for cellular survival, but the eukaryotic genome constantly incurs modifications that are potentially cytotoxic or mutagenic. Predicted increases in solar UV-B radiation have served to focus attention on the cytotoxic effects of UV-B on plants. One of the reasons that UV-B radiation is potentially so damaging is that it can be absorbed by a wide range of biologically important molecules. These include proteins, quinones, lipids and most significantly nucleic acids. Such non-specific absorption would almost certainly have deleterious consequences. In addition, this could not provide the regulatory potential to allow the plant to adjust to the UV-B environment (Jordan, 1996). Plants may, however, "sense" their UV-B environment by either UV-B photoreceptors alone or in conjunction with other photoreceptors detecting various regions of the solar spectrum (see section 1.2.1.1).

The DNA photoproducts formed by UV-B radiation can be divided into two classes, the dimeric photoproducts that make up the bulk of the lesions formed after UV irradiation, and monomeric photoproducts. Cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidinone photoproducts (or (6-4) photoproducts) make up the majority of UV-induced DNA damage products and both are toxic lesions. CPDs

are formed when adjacent pyrimidines become covalently linked by the formation of a four-membered ring upon UV exposure and have a stable confirmation which is resistant to pH and temperature extremes (Mitchell and Nairn, 1989; Taylor *et al.*, 1997). (6-4) Photoproducts are formed at positions of cytosine 3` to pyrimidine nucleosides and produce alkali-labile lesions.

At the molecular level, pyrimidine dimers are known to inhibit the progress of microbial and mammalian DNA polymerases. Since pyrimidine dimers cannot effectively base pair with other nucleotides, they are not directly mutagenic, but instead act as blocks to DNA replication and transcription as RNA polymerases have been reported to stall at the site of these photoproducts (Britt, 1997). Unrepaired dimers are lethal to the cells because they deform the DNA helix, interfere with both replication and transcription. Thus a single pyrimidine dimer, if left unrepaired, is sufficient to completely eliminate the expression of a transcriptional unit. If every pyrimidine dimer acts as a block to transcription and replication, while only a fraction of dimers result in mutation, the inhibitory effects of UV on transcription and DNA replication are probably more significant in terms of plant growth than its mutagenic effects.

4.1.1 Study approach

Physiological studies were conducted on populations of *D. sinuata* derived from a common seed stock. The plants were exposed concurrently over four successive generations to either ambient (representing no stratospheric ozone depletion) or elevated (representing 20% stratospheric ozone depletion) UV-B levels (see Chapter 3). Results pointed to the possibility of a cumulative effect of UV-B radiation on *D. sinuata* which could be heritable as previously irradiated plants grown in the absence of UV-B in the greenhouse have been found to show significant differences when compared to control plants. Among these was diminished photosynthetic rate (a consequence of a reduced leaf density), and diminished foliar levels of carotenoids, polyphenolics and anthocyanins. Altered physiology was accompanied by reduced apical dominance and earlier flowering, increased branching and inflorescence production and greater partitioning of biomass to reproductive structures, and diminished seed production (Musil, 1996). Although reproductive organs are

generally considered to be well protected from UV-B during developmental and maturation phases, long term exposure to high UV-B levels may also affect the reproductive structures of plants and cause DNA damage. Mature pollen grains are potentially very susceptible to UV-B induced DNA damage during the short period between anther dehiscence and pollen tube penetration into stigma tissues (Jackson, 1987). It is therefore proposed that radiation damage to DNA might be transmitted via the seeds and pollen (Mulcahy, 1971, 1974) and result in morphological, physiological and genetic changes in the plants.

The genetic analysis employed in this study therefore involves investigations of *D. sinuata* chromosomal (this chapter) and chloroplast DNA (chapter 2) for evidence of UV-induced mutagenesis. Since UV-B is known to affect adjacent pyrimidine bases on the DNA by linking them to form dimers, it was postulated that an assay that targeted pyrimidine dimers would aid in the detection of DNA mutations directly linked to UV-damage. Therefore, genetic analysis of pyrimidine dimer (T=T) formation in test plants was conducted using the *Dra*I assay method of Harlow *et al.* (1994) (see section 4.1.3). This assay was developed for comparing induction of UV damage in a *uvh1* (an *Arabidopsis* mutant hypersensitive to UV light and ionising radiation) and wild-type plants. The authors used the assay to determine whether DNA was less protected from UV damage in *uvh1* plants than in wild-type plants. In this study, the *Dra*I assay was used not only to look for unrepaired dimers, but would also reflect mutated *Dra*I sites where the damage in the previous generations had been inaccurately repaired. Exposure of plants to UV-B radiation would create mutation hotspots at the site of dimer formation if such damage were not repaired accurately.

The first part of this study describes the cloning, sequencing and characterisation of the gene coding for the large subunit (*rbc*L) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) from *D. sinuata*. This gene was used as a probe in the *Dra*I assay and later in northern blot analysis (see Chapter 5). The isolation of the *18S-rDNA* probe used in the *Dra*I assay and as an internal control in northern blot analysis is also described.

4.1.2 Rubisco and its role in photosynthesis

Rubisco is the key enzyme of photosynthesis in all plants and algae and in most photosynthetic bacteria and is responsible for the fixation of carbon dioxide in the Calvin Cycle (Clegg, 1993). The enzyme, which is located in the stroma of chloroplasts, comprises up to 50% of leaf proteins and is the most abundant protein in the biosphere. During photosynthesis, one molecule of CO_2 reacts with and carboxylates one molecule of D-ribulose 1,5-bisphosphate to yield two molecules of 3-phospho-D-glycerate during the Calvin cycle (Peters and Silverthorne, 1995). Rubisco thus sits at the nexus of the earth's carbon cycle.

The Calvin cycle represents a cyclic set of reactions that occurs in chloroplasts in the majority of plants resulting in the fixation of CO_2 as glucose using the ATP and NADPH formed in the light reactions of photosynthesis (Palmer, 1996). ATP phosphorylates the two molecules of phosphoglycerate from the carboxylation reaction to 1,3-bisphosphoglycerate, which in turn is reduced by NADPH to glyceraldehyde 3-phosphate. Aldolase, transketolase and other enzymes can then convert this into a hexose product (fructose-6-phosphate) and ribulose 5-bisphosphate. ATP then phosphorylates the latter to ribulose 1,5-bisphosphate, completing the cycle.

The great abundance of Rubisco in plants has opened up many opportunities for scientists to study and understand various cellular processes owing to its central importance in photosynthetic carbon fixation and the early technical advantages associated with the study of the chloroplast genome. The enzyme and its encoding genes have served as models for elucidating fundamental properties of plant gene regulation, chloroplast-nuclear co-ordination, and protein import and assembly (Gutteridge and Gatenby, 1995). In higher plants, the Rubisco holoenzyme is a 16-mer structure comprised of eight identical catalytic polypeptides of 50 000 to 60 000 daltons (LSUs) and eight smaller peptides of 12 000 to 20 000 daltons (SSUs) (Hanley-Bowdoin *et al.*, 1985; Clegg, 1993). The large subunit (LSU) is encoded on the chloroplast genome and is translated on 70S chloroplast ribosomes. Under most conditions, the synthesis of the subunits is co-ordinated and regulated by light and cytokinins.

The *rbc*S genes are located in the nucleus and constitute a small multigene family of at least four members, depending on the plant species (Peters and Silverthorne, 1995). They may have a few introns, encode mature proteins of approximately 120 amino acids and are more divergent than the chloroplast encoded *rbc*L genes. The individual family members are nearly identical, whereas the non-coding regions of their mRNAs are more variable in sequence, and it is assumed that resultant polypeptides function with equal efficiency. The small subunit protein is encoded in the nucleus and is translated on free, cytoplasmic ribosomes as a large precursor polypeptide (20 kDa) which is cleaved to the mature form during import into the chloroplasts (Berry *et al.*, 1985). After translation in the cytoplasm on 80S ribosomes, the mature S-subunits are imported into chloroplasts and after processing, they are assembled with plastid synthesised L-subunits into the functional L_8S_8 Rubisco holoenzyme. It is, therefore, likely that the large number of redundant *rbc*S sequences compensate for the multiple copies of the chloroplast genome, each containing a single *rbc*L gene, thereby allowing production of large amounts of the holoenzyme. Despite this apparent disparity in gene copy number, approximately equal amounts of LSU and SSU mRNA and protein accumulate, suggesting co-ordinate expression of these chloroplastic and nuclear genes (Peters and Silverthorne, 1995).

Due to the prokaryotic origin of the chloroplast genome, the transcriptional and translational recognition sequences of many chloroplast genes resemble those found in prokaryotic organisms such that chloroplast *rbc*L genes can easily be expressed in *Escherichia coli* (Shinozaki *et al.*, 1986a,b). Transcriptional initiation rates from the *rbc*L promoter are not only influenced by the promoter sequence but are also modified by the nearby *atp*B promoter. It has been shown that in chloroplast genomes of higher plants, the genes for the large subunit of Rubisco and the beta subunit of ATP synthetase (*atp*B) are adjacent and transcribed divergently (Hanley-Bowdoin *et al.*, 1985). The two promoters are positioned approximately 100 bp apart in opposite orientation resulting in divergent transcription (Gutteridge and Gatenby, 1995). The two promoters do not function independently because the binding of RNA polymerase at the *rbc*L promoter has been found to interfere with binding and transcription from the *atp*B promoter by steric hindrance at the two RNA polymerase binding sites. This mutual interference may be a control mechanism to regulate different levels of expression in chloroplasts and was found to be eliminated by

deletion of one promoter or increasing the distance between them (Gutteridge and Gatenby, 1995).

4.1.3 The *Dra*I Assay

The principal UV-photoproducts in DNA are cyclobutane pyrimidine dimers (CPDs) and pyrimidine - (6-4')- pyrimidone photoproducts [(6-4') adducts]. UV radiation affects adjacent thymine bases of DNA, linking them together to form dimers which block restriction endonuclease recognition or cleavage for those enzymes that recognise sites containing adjacent thymines. The *Dra*I assay looks at evidence of residual UV-B-induced mutations in genomic DNA in sequences flanking the probed region. This assay is based on Whittaker and Southern's (1986) finding that restriction enzyme activity can be inhibited by the presence of DNA damage at the recognition sequence. Since any alteration of the bases within this recognition sequence would be expected to inhibit cleavage, UV irradiation of substrate DNA should destroy potential cleavage sites. Partial DNA digests therefore result when UV-irradiated DNA is digested with enzymes whose recognition sequences contain adjacent thymidines.

Any DNA sequence having adjacent thymines is, therefore, a potential UV-B target. When a thymine dimer is produced in a recognition site for a given enzyme, this site is not cleaved. Therefore, any effect of irradiation at UV-B wavelengths on the substrate properties of DNA should be most pronounced for those endonucleases which recognize sequences containing adjacent thymines such as *Dra*I (TTTAAA) (Hall and Larcom, 1982; Harlow *et al.*, 1994). In addition, if a site originally contained a dimer, this could lead to a mutation at that site if the lesion is incorrectly repaired. As a result, that site would not be recognized either. Because the *Dra*I recognition sequence contains a minimum of four potential sites for induction of CPD or 6-4 photoproducts, two on each strand, inhibition of *Dra*I activity by UV damage should provide an assay for CPD or 6-4 photoproduct induction (Harlow *et al.*, 1994) or UV-B-induced mutations. The two fragments that would have been produced by cleavage at this site now appear as one piece of DNA with a higher molecular weight. Upon electrophoresis, this uncleaved piece appears as a new band that migrates more slowly than either of its constituent bands.

To determine the dimer content of *Dra*I sites or UV-B-induced mutations in specific DNA fragments, suitable hybridisation probes are used, in this case *18S* rRNA and *rbc*L genes. Since the probe sequences do not contain *Dra*I sites, only one band is to be expected following complete digestion of plant DNA with *Dra*I (Harlow *et al*, 1994). The appearance of partially digested bands is a function of UV fluence and amount of dimers present in the DNA or UV-B induced mutations at dimer sites. The proportion of damaged molecules thus represents the average frequency of dimer production in one or more of *Dra*I sites that flank the probed region. The advantage of this assay lies in the fact that only specific sites flanking the gene of interest are being investigated unlike other methods which look at all regions of the genome. Examples of such methods are amplified fragment length polymorphisms (AFLPs), restriction fragment length polymorphisms (RFLPs) and Random amplified polymorphic DNA sequences (RAPDs). These methods suffer the main drawback in that they tend to be too general.

The 18S rRNA *gene*

The genes that specify the cytoplasmic ribosomal RNA (rRNA) of higher eukaryotes are arranged as long tandem repeats of highly similar but not identical repeat units in the nuclear DNA. Each repeat unit encodes a precursor RNA molecule which is processed into the *18S, 5.8S* and *25S* as well as an intergenic non-transcribed spacer (NTS) sequence. The *18S, 5.8S* and *25S* genes are clustered and are transcribed as one unit (Campbell *et al,* 1992; Rogers and Bendich, 1987). The NTS is a source of length heterogeneity, usually resulting from the variation in the number of short (100-300 bp) elements (subrepeats) arranged in tandem within the NTS (Jorgensen *et al.,* 1987). In the pea genome, approximately 4000 copies of the rDNA repeat have been reported to show considerable heterogeneity with respect to the length of the NTS region, and differences are also frequently observed between different genotypes. In both cases, the length variation is reported to appear due primarily to differences in the number of subrepeat elements (Jorgensen *et al.,* 1987).

The rbcL gene

The *rbc*L gene is currently being used to examine evolutionary relationships in a wide diversity of plant groups, and several studies have demonstrated the potential of the *rbc*L for resolving phylogenetic relationships at the interfamilial level (Kim *et al.*, 1992; Clegg, 1993). The *rbc*L gene is present as a single copy on the multicopy chloroplast genome, but the actual *rbc*L copy number per cell can be high due to the fact that many copies of the chloroplast genome are present per plastid. In chloroplast genomes which have the typical inverted repeat structure, the *rbc*L gene is located in the large single copy region next to, and of opposite polarity from polycistronic genes encoding the beta- and epsilon-subunits of the chloroplast ATPase (*atp*B/E) (Manzara and Gruisemm, 1988). In all higher plants studied to date, the *rbc*L coding sequence is continuous, approximately 1.4 kb in length and does not contain introns except for rare exceptions and encodes a polypeptide of approximately 475 amino acids (Gutterridge and Gatenby, 1995).

The coding sequences are highly conserved between *rbc*L genes of different species, with the majority of nucleotide changes occurring at the 3' ends of the genes. In addition many of the base changes are silent, resulting in even higher similarity between the amino acid sequences (Manzara and Gruissem, 1988). For instance, the homology between the *rbc*L nucleotide sequences of maize versus spinach is 84%, while the similarity between the corresponding amino acid sequences is 90%.

In this study, plants were grown in the absence of UV-B radiation, and the aim of this chapter was to assay for evidence of UV-B induced mutagenesis. A further aim was to clone and characterise the *rbc*L gene. The *rbc*L gene from *D. sinuata* has not been cloned or characterised previously.

4.2 Materials and methods

4.2.1 Isolation and quantitation of total plant DNA

All standard DNA manipulations were performed as described by Sambrook *et al.* (1989), with some minor modifications, and according to specifications of the

manufacturers and suppliers of the DNA modifying enzymes (Roche Diagnostics GmBH, Amersham or Promega).

Plant material from both Generation 0 and Generation 3 were used. Generation 0 material was used to show if growing plants in the greenhouse as controls (generation 3A) had any stressful influence on the plants. When this part of the study was conducted, seed material for generations four and five were not available, hence the generation three plants were used for analysis. Fresh plant material (100 mg) from *D. sinuata* plants was ground to a fine free flowing powder in a mortar in the presence of liquid nitrogen. The powder was transferred to an eppendorf tube and 750 µl of extraction buffer (100 mM Tris, 50 mM EDTA, 0.5 M NaCl, 10 mM β-mercaptoethanol) and 50 µl of 20% SDS (w/v) were added. This was mixed thoroughly by vortexing. Ten µl of RNAse A (10 mg/ml) was added, and the tube was shaken to mix the contents. The tubes were incubated at 65°C in a water bath for 10 minutes. The mixture was then emulsified in an equal volume of phenol/chloroform and centrifuged at 14 000 rpm for 10 minutes. The aqueous phase was then extracted with an equal volume of chloroform, centrifuged for 10 minutes at 14 000 rpm. One-tenth volumes of 3 M sodium acetate, pH 5.2 was added to the aqueous phase which was then precipitated with 2.5 volumes of ice cold ethanol. The DNA pellet was washed with 70% ethanol and resuspended in TE buffer (pH 8.0). DNA concentrations were determined by measuring the absorbance at 260 nm, or by fractionating DNA aliquots on a gel against known concentrations of λ-DNA

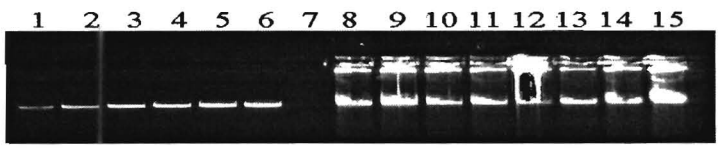

Figure 4.1 Genomic DNA as seen on a 0.8% agarose gel. Lanes 1 – 6 lambda DNA standards of varying concentrations (lane 1 = 10 ng, lane 2 = 20 ng, lane 3 = 30 ng, lane 4 = 40 ng, lane 5 = 50 ng and lane 6 = 60 ng). Lane 7 = blank. Lanes 8 – 15 represent undigested genomic DNA from various samples.

standards (see Figure 4.1), and quantifying densitometrically using the Gel Trak software programme (D. Maeder, unpublished).

4.2.2 The *18S* rRNA and *rbc*L probes

Both chloroplast- (*rbc*L) and nuclear-encoded (*18S* rRNA) probes were used in the assay. The probes were generated by the polymerase chain reaction (PCR) from seedling genomic DNA of *D. sinuata* (Figure 4.2). The DNA used for making probes was isolated from plants from Generation 0, the parental seed stock from which all experimental material was derived (see Chapter 3, Figure 3.1).

4.2.2.1 Construction of the *18S* rRNA probe

Oligonucleotide primers designed to complement an internal fragment of the *18S* rRNA gene sequence from *Arabidopsis thaliana* (Unfried *et al.,* 1989) were used to produce a 1.6-kb fragment of the *18S* rRNA gene. The sequence of the forward primer (primer 1) was 5'- GTG TAA GTA TGA ACG AAT TC–3', and that of the reverse primer (primer 2) was 5'– GGAATT CTT CGT TGA AGA CC–3'. The PCR conditions were as follows: 70 ng template DNA; 5 µl of 10 µM primer 1; 5 µl of 10 µM primer 2; 4.0 µl of 5 mM dNTPs; 8 µl of 25 mM $MgCl_2$; 10 µl 10X PCR buffer; 0.5 µl Taq polymerase (5U/µl). Roche Diagnostics GmBH, Mannheim, Germany, supplied reagents used for PCR. The total reaction volume was made up to 100 µl with distilled H_2O, and then overlaid with mineral oil to eliminate evaporation. The PCR cycle profile was as follows: initial denaturation was at 94°C for 60 seconds, followed by 30 cycles of denaturation at 94°C for 60 seconds, primer annealing at 50°C for 30 seconds, and PCR product extension at 72°C for 90 seconds, followed by a final extension at 72°C for 5 minutes. PCR products were visualized by running 2 µl of the reaction mixture on a 1% agarose gel.

4.2.2.2 Construction of the *rbc*L probe

A 1.1-kb fragment of the *D. sinuata rbc*L gene was amplified from genomic DNA by PCR (see Figure 4.2). Oligonucleotide primers designed to complement an internal

fragment of the *rbc*L sequence from the grain amaranth, *Amaranthus hypochondriacus* (Michalowiski *et al.,* 1990) were used. The sequence of the forward *rbc*L primer (primer 1) was 5'-GAT ATC TTG GCA GCA TTC CG–3', and that for the reverse primer (primer 2) was 5'–TGT CCT AAA GTT CCT CCA CC–3'. The PCR conditions and visualization were as for the *18S* rRNA probe (see 4.2.2.1).

4.2.2.3 Cloning and sequencing of the *rbc*L and *18S* rRNA PCR products

Both PCR products were fractionated in a 1% agarose gel by electrophoresis and the fragments were excised and purified using the Geneclean II® Kit according to the manufacture's protocol (BIO 101 Inc. La Jolla, USA). These were then cloned into the *Eco*RV site of pSK (p-Bluescript) by blunt end ligation and named prbcL and p18S for the *rbc*L and *18S* rRNA fragments respectively. The authenticity of the two cloned *rbc*L and *18S* rRNA gene fragments was determined by end sequencing. DNA sequencing was done by the dideoxy chain-termination method of Sanger *et al.,* (1977) with a sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech). This method is utilised for sequencing clones in M13 based vectors. All reactions were performed according to the manufacturer's instructions and cycle sequenced using the GeneAmp PCR System 9700 amplifier (Perkin Elmer Applied Biosystems). The data was processed by ALFwin version 2.1 software, (Amersham Pharmacia Biotech) and homology searches and sequence analysis was done using the BLAST programme (Altschul *et al*, 1997).

4.2.2.4 Labelling of probes and Southern hybridization

The probes used for detection were DIG-labelled either by the random-priming method or through PCR incorporation according to the supplier's protocol (Roche Diagnostics GmBH, Mannheim, Germany). All Southern hybridization steps were done according to standard procedures (Sambrook *et al.,* 1989). Hybridizations were done at 42°C overnight in DIG-Easy Hyb solution according to the supplier's protocol (Roche Diagnostics GmBH, Mannheim, Germany). At the end of the hybridisation, the membrane was washed twice, 5 minutes per wash in 2X wash solution (0.3 M

NaCl, 30 mM sodium citrate; pH 7.0, containing 0.1% SDS (w/v)) at room temperature. After these low stringency washes, high stringency washes were carried out by washing the membrane twice, 15 minutes per wash in 0.1X wash buffer (15 mM NaCl, 1.5 mM sodium citrate; pH 7.0, containing 0.1% SDS (w/v)) (prewarmed to 65°C) at 65°C.

After the post-hybridisation washes, membranes were treated with blocking reagent for 30 minutes to prevent non-specific attraction of the antibody to the membrane. Membranes were then incubated with a dilution of anti-digoxigenin Fab fragments conjugated to alkaline phosphatase for 30 minutes (Roche Diagnostics GmBH, Mannheim, Germany) followed by two washes in Buffer 1 (100 mM maleic acid, 150 mM NaCl; pH 7.5) + 0.3% Tween® 20 for 15 minutes each at room temperature. The membranes carrying the hybridised probe and bound antibody conjugate were finally equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl; pH 9.5) for two minutes, before being reacted with the chemiluminescent substrate, CSPD and exposed to X-ray film to record the chemiluminescent signal.

4.2.3 Cloning the *rbc*L gene

Genomic DNA from Generation 0 plants was digested with various restriction enzymes (*Hin*dIII, *Bam*HI, *Eco*RI, *Eco*RV, *Pst*I, *and KpnI*) and probed with the DIG-labelled *rbc*L probe. A putative 7.51-kb *Bam*HI fragment containing the *rbc*L gene was identified. This was cloned into the *Bam*HI site of pSK and the construct was called pDsrbcl. This construct was digested with several enzymes (*Bam*HI, *Bgl*II, *Bst*XI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sph*I, and *Sty*I) and again probed with the DIG-labelled *rbc*L probe. Fragments that hybridised to the *rbc*L gene probe and were small enough to be sequenced were selected for subcloning and sequencing.

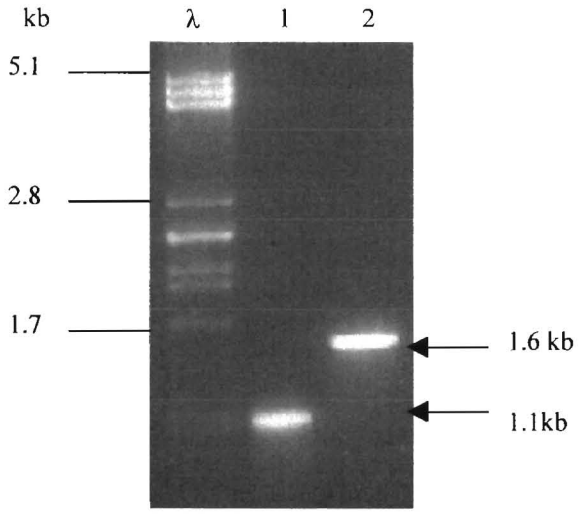

Figure 4.2. PCR products used as probes in the study. Lane 1 = *rbc*L, lane 2 = 18S rDNA. The two PCR products were 1.6kb (18S rDNA) and 1.1 kb (*rbc*L). Lambda DNA digested with *Pst*I (lane λ) was used as molecular weight standard. The sizes of the different fragments are shown on the right.

4.2.4 *Dra*I assay

DNA was isolated from the original seed stock (Generation 0), and control and test plants from Generation 3. Equal amounts (2 μg) of DNA were routinely digested to completion with *Dra*I overnight, separated by electrophoresis and then transferred to positively charged nylon hybridisation membranes (Hybond+) (Roche Diagnostics GmBH, Mannheim, Germany) according to standard procedures (Sambrook *et al.*, 1989). The blots were hybridised to a specific DIG-labelled *rbc*L or *18S* rRNA probe (Hoeltke *et al.*, 1995). The hybridization and detection procedures were carried out as described in section 4.2.2.4.

4.3 Results

4.3.1 Construction of the *18S* rRNA and *rbc*L gene probes

The *18S* rRNA gene sequence was verified by end-sequencing approximately 400 bp of the 3'-end of the cloned PCR product (plasmid p18S). The *18S* rRNA gene sequence was found to be >95% identical to the reported *A. thaliana 18S* rRNA gene (Unfried *et al.*, 1989). The *rbc*L fragment (prbcL) was sequenced from both ends and

was found to have over 98% identity to a reported sequence of *D. pluvialis*, a close relative at the nucleotide level (Kim *et al.*, 1992).

4.3.2 Cloning of the *rbc*L gene

When pDsrbcL was digested with *Bst*XI, it released five fragments, three of which hybridized to the *rbc*L probe: a 1.6-kb, 1.0-kb and a 0.7-kb fragment (Figure 4.3, lane 3). The 1.0-kb fragment was cloned into the *Bst*XI site of pSK (designated pBxt) and sequenced successfully (Figure 4.4).

Restriction enzyme digestion of the plasmid pDsrbcL with *Bgl*II produced a 2-kb fragment that hybridised with the *rbc*L gene probe (Figure 4.3, lane 2). This fragment was cloned into the *Bam*HI site of pSK and end sequenced. It was designated pBglII. The 5' end of this construct showed high identity (88%) at the nucleotide level to the reported sequence of the *Nicotiana plumbaginefolia* chloroplast *atp*B gene for ATPase subunit β, and the *atp*B/*rbc*L spacer from *Bellis perennis*, also an Asteraceae

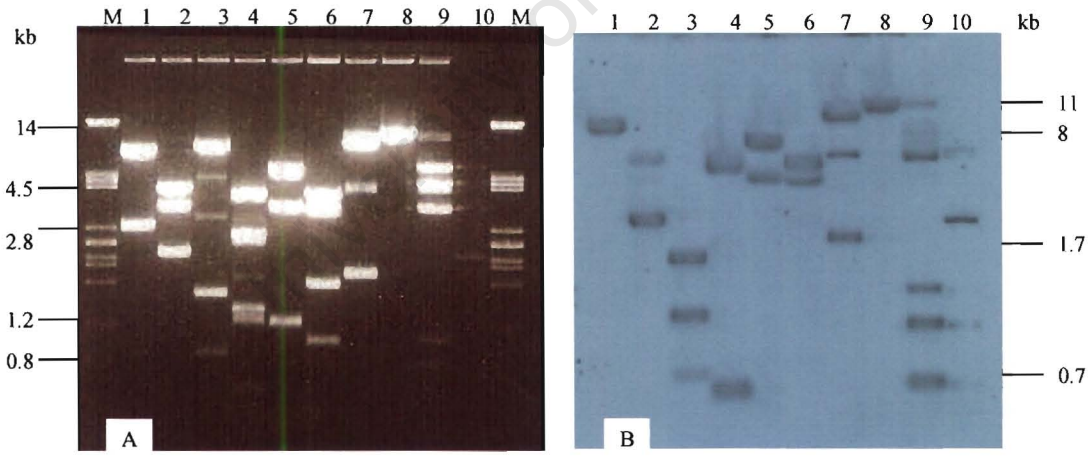

Figure 4.3 Restriction enzyme digestion and southern hybridization of pDsrbcL. Panel A = restriction endonuclease digest of pDsrbcL and B = southern blot. Fragments of the *rbc*L gene were localised by probing various digests of plasmid pDsrbcL with a DIG-labelled *rbc*L probe (panel B). M = molecular weight markers (λ *Pst*I), lanes: 1, *Bam*HI; 2, *Bgl*II; 3, *Bst*XI; 4, *Eco*RI; 5, *Hin*dIII; 6, *Kpn*I; 7, *Pst*I; 8, *Sph*I; 9, *Sty*I; 10, uncut DNA (positive control).

(Altschul, 1997).

Restriction mapping of the pBglII sequence identified two *Pst*I sites in this construct. The pBglII construct was therefore digested with *Pst*I, releasing a 1.1-kb and a 0.9-kb

*Pst*I fragment.The 1.1 kb fragment was cloned into the *Pst*I site of pSK (to give pSw) and sequenced. The 0.9 kb fragment was re-ligated to give plasmid pMpo and sequenced. The different constructs are represented in Figure 4.4 showing the sequencing strategy for the *rbc*L gene. The fragment shown in green (the plasmid

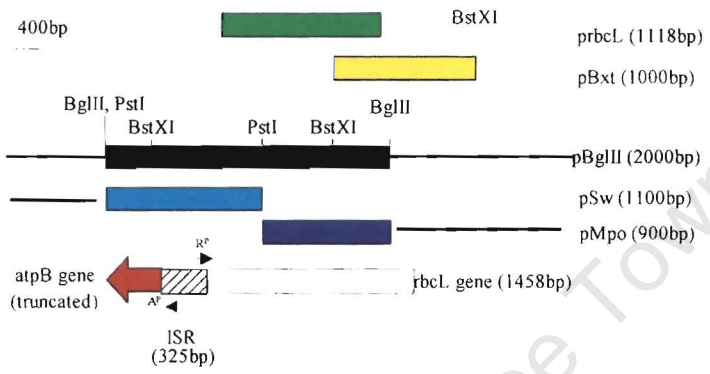

Figure 4.4 Cloning strategy for the *rbc*L gene. The different constructs that were made in order to sequence the entire gene are shown. The sizes of the respective fragments are indicated in base pairs, and restriction sites are also indicated. ISR = short intergenic spacer region for the *atp*B/*rbc*L spacer. The thin lines = pSK Bluescript plasmid DNA. Thick coloured lines = *D. sinuata* ctDNA. R^p and A^p = *rbc*L and *atp*B promoters respectively

prbcL) represents the internal fragment of the *rbc*L gene that was amplified by PCR.

Homology searches were done against all the clones sequenced, and against the previously sequenced *rbc*L PCR product and a reported sequence of *D. pluvialis* (Kim *et al.*, 1992). Several regions of sequence overlap were found and these were used to cross-check the *rbc*L sequence and to align the entire sequence (Figure 4.4). The nucleotide and deduced amino acid sequence of the *D. sinuata rbc*L gene is shown in Figure 4.5 with the flanking upstream region representing part of the *atp*B/*rbc*L spacer region and a truncated *atp*B gene. The flanking down-stream (bases 1575-1663 in Figure 4.5) sequence is also indicated. This showed high homology at the nucleotide level to the uncharacterised ORF512 region of the *Nicotiana tabacum* chloroplast genome (Shinozaki *et al.*, 1986a). The *rbc*L gene was 1458 bp long with an open reading frame of 486 amino acids. The nucleotide sequence from *D. sinuata* was compared with the *rbc*L gene sequence from *D. pluvialis*. The two had over 98% identity (Figure 4.5) at the nucleotide level. Twenty-six nucleotides differed between

the two, but only four amino acids from a total of 486 amino acids were different.

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CAACATATATCACTGCCAAGAGGGAATTTCTTAGTATTTGGGCAATTTTTTGGTATTTCAATTCAAAAAAAAATA      -375
AGAAGTGGGTTGCGCCATATATATGAAAGAGTATACAATAATGATGTATTTGCCGAATTAAATAACATGGTCTAA      -300
TAATAAAGCATTCTGATTAGTTGATAATTTTACTATTAGTTGGGAATTTTGTGAAAGGTTCCTGTAAAAAGTTTC      -150
ATTAACGCCTAATTCATGTCGAGTAGACCTTGTTGTTGTGAGAATTCTTAATTCATGAGTTGTAGGGAGGGATTT       -75
ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGATTATAAATTGACTTATTAT        75
 M  S  P  Q  T  E  T  K  A  S  V  G  F  K  A  G  V  K  D  Y  K  L  T  Y  Y        25
ACTCCTGACTATAAAACCAAGGATACTGATATCTTGGCAGCATTCCGAGTAACTCCGCAACCTGGAGTTCCGCCT      150
 T  P  D  Y  K  T  K  D  T  D  I  L  A  A  F  R  V  T  P  Q  P  G  V  P  P        50
GAAGAAGCAGGGGCTGCAGTAGCTGCCGAATCTTCTACTGGTACATGGACAACCGTGTGGACCGATGGACTTACG      225
 E  E  A  G  A  A  V  A  A  E  S  S  T  G  T  W  T  T  V  W  T  D  G  L  T        75
AGCCTTGATCGTTACAAAGGGAGATGCTATGGAATCGAGGCTGTTCTAGGAGAAGAAAATCAATATATTGCGTAT      300
 S  L  D  R  Y  K  G  R  C  Y  G  I  E  A  V  L  G  E  E  N  Q  Y  I  A  Y       100
GTAGCTTACCCATTAGACCTTTTTGAAGAAGGTTCCGTTACTAACATGTTTACTTCCATTGTAGGTAATGTATTT      375
 V  A  Y  P  L  D  L  F  E  E  G  S  V  T  N  M  F  T  S  I  V  G  N  V  F       125
GGGTTCAAAGCCCTGCGTGCTCTACGTCTGGAAGATTTGCGAATTCCTACTGCGTATGTTAAAACTTTCCAAGGT      450
 G  F  K  A  L  R  A  L  R  L  E  D  L  R  I  P  T  A  Y  V  K  T  F  Q  G       150
CCGCCTCACGGCATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGGCGTCCCCTGTTGGGATGTACTATTAAA      525
 P  P  H  G  I  Q  V  E  R  D  K  L  N  K  Y  G  R  P  L  L  G  C  T  I  K       175
CCTAAATTGGGGCTATCCGCTAAAAACTACGGTAGAGCTGTTTATGAATGTCTTCGTGGTGGCCTTGATTTTACT      600
 P  K  L  G  L  S  A  K  N  Y  G  R  A  V  Y  E  C  L  R  G  G  L  D  F  T       200
AAAGATGATGAGAACGTGAACTCCCAACCATTTATGCGTTGGAGAGACCGTTTCTTATTTTGTGCCGAAGCTATT      675
 K  D  D  E  N  V  N  S  Q  P  F  M  R  W  R  D  R  F  L  F  C  A  E  A  I       225
TATAAAGCACAAGCCGAAACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCGGGTACATGCGAAGAAATG      750
 Y  K  A  Q  A  E  T  G  E  I  K  G  H  Y  L  N  A  T  A  G  T  C  E  E  M       250
ATGAAAAGGGCTGTATTTGCCAGAGAATTGGGAGTTCCTATCATAATGCATGACTACCTAACAGGGGGATTCACT      825
 M  K  R  A  V  F  A  R  E  L  G  V  P  I  I  M  H  D  Y  L  T  G  G  F  T       275
GCAAACACTAGCTTGGCTCATTATTGCCGAGATAATGGCCTACTTCTTCACATCCACCGCGCAATGCATGCAGTT      900
 A  N  T  S  L  A  H  Y  C  R  D  N  G  L  L  L  H  I  H  R  A  M  H  A  V       300
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 I  D  R  Q  K  N  H  G  I  H  F  R  V  L  A  K  A  L  R  M  S  G  G  D  H       325
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 I  H  S  G  T  V  V  G  K  L  E  G  E  R  E  I  T  L  G  F  V  D  L  L  R       350
GATGATTTTATTGCCCAAGATGCAAGTCGCGGTATTTATTTCGACCAAGATTGGGTTTCTCTACCAGGTGTTCTG     1125
 D  D  F  I  A  Q  D  A  S  R  G  I  Y  F  D  Q  D  W  V  S  L  P  G  V  L       375
CCGGTAGCTTCGGGAGGTATTCACGTTTGGCATATGCCTGCTCTGACCGAGATCTTTGGCGATGATTCCGTACTA     1200
 P  V  A  S  G  G  I  H  V  W  H  M  P  A  L  T  E  I  F  G  D  D  S  V  L       400
CAGTTCGGTGGAGGAACTTTAGGGCACCCTTGGGGAAATGCACCTGGTGCTGTAGCTAATCGAGTGGCTCTAGAA     1275
 Q  F  G  G  G  T  L  G  H  P  W  G  N  A  P  G  A  V  A  N  R  V  A  L  E       425
GCATGTGTACAAGCTCGTAACGAGGGACGCAATCTTGCTACTCAGGGTAATGAAATTATCCGTGAGGCTGCCAAA     1350
 A  C  V  Q  A  R  N  E  G  R  N  L  A  T  Q  G  N  E  I  I  R  E  A  A  K       450
TGGAGTCCTGAACTAGCTGCTGCTTGTGAAGTATGGAAGGAGATCAAATTTGAATTTCAGGCAATGGATACTTTG     1425
 W  S  P  E  L  A  A  A  C  E  V  W  K  E  I  K  F  E  F  Q  A  M  D  T  L       475
GATACGGATAAGGATAAGGATAAGAAGAGATAACGTTCGTTCTCTTAATTGAATTTCAATGAAATTYGGCCCAAT     1500
 D  T  D  K  D  K  D  K  K  R  *                                                  486
CTTTTACTAAAAGGATTGAGCCGAATCCAACATGCTTTAGATATATAAATACTTATATAGATATAGAAAATTTCA      1575
ACTAAAAACTCGAAGACTAAACAACTCAAATCTTTCTATTTTTGTGTTGATCACTAGTGTCTAGAGCGCCACCGC     1650
GGTGGCGTTTTGC                                                                    1663
```

Figure 4.5 Nucleotide and deduced amino acid sequence of the *D. sinuata rbc*L gene. The start and stop codons are represented in **bold**. The underlined amino acids are those that differ from the closely related *D. pluvialis* sequence. The untranslated upstream sequence represents part of the atpB/rbcL spacer region. The open reading frame from 1-1458 encodes 486 amino acids with an apparent molecular weight of 53617 daltons.

A computer search using the BLAST network service revealed that the *rbc*L gene is highly conserved. The *D. sinuata rbc*L nucleotide sequence was compared with *rbc*L sequences of six other plants, namely *Felicia bergeriana, Cichorium intybus* L, *D. pluvialis, Helianthus annuus, Flaveria pringlei* and *Amaranthus hypochondriacus* which showed the highest homology. A putative homology tree was constructed from the seven sequences (Figure 4.6).

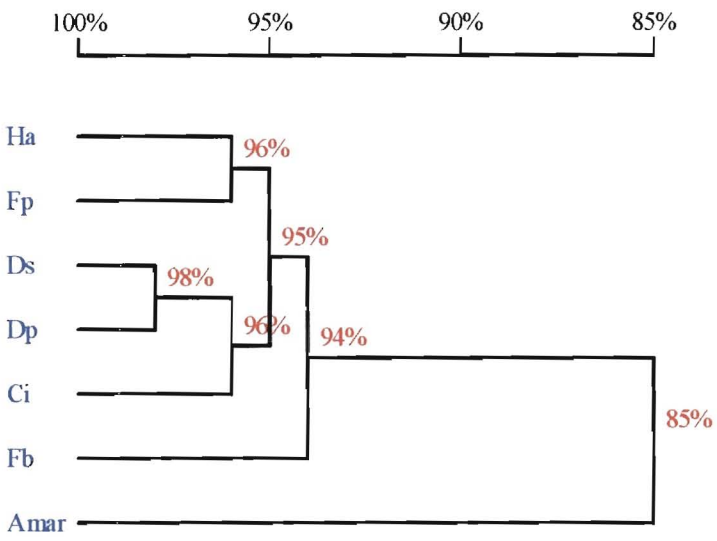

Figure 4.6 Homology tree of the *rbc*L sequences from seven different species. Fb = *Felicia bergeriana*; Ci = *Cichorium intybus* L; Ds = *D. sinuata;* Dp = *D. pluvialis;* Ha = *Helianthus annuus;* Fp = *Flaveria pringlei; and* Amar = *Amaranthus hypochondriacus*. The percentage value is the homology level between the species at the nucleotide level.

4.3.3 The *Dra*I assay

Gels were blotted overnight and probed with either a DIG-labelled *18S* rRNA or *rbc*L probe (Figures 4.7 and 4.8 respectively). The *18S* rRNA probe hybridised to several high molecular weight bands ranging in size from 7 to 11.5 kb (Figure 4.7). Hybridizing the same blot with the *rbc*L probe gave a single intense band of approximately 7.51 kb (Figure 4.8). A total of 101 DNA samples from individual seedlings were analysed. These consisted of 45 samples from the original seed batch (see Chapter 3, Figure 3.1; herein referred to as generation zero or G0), 22 G3A plants (representing three generations of ambient UV-B irradiation) and 34 G3H plants (three generations of enhanced UV-B irradiation. A total of 30 DNA samples isolated from plants grown from the original seed batch (G0 or control plants) and another 30 from the enhanced UV-B group (G3H) were digested with *Dra*I and probed with the *rbc*L gene. Genomic DNA from both enhanced UV-B group and control samples always showed a single intense band of approximately 7.5 kb (Figure 4.8).

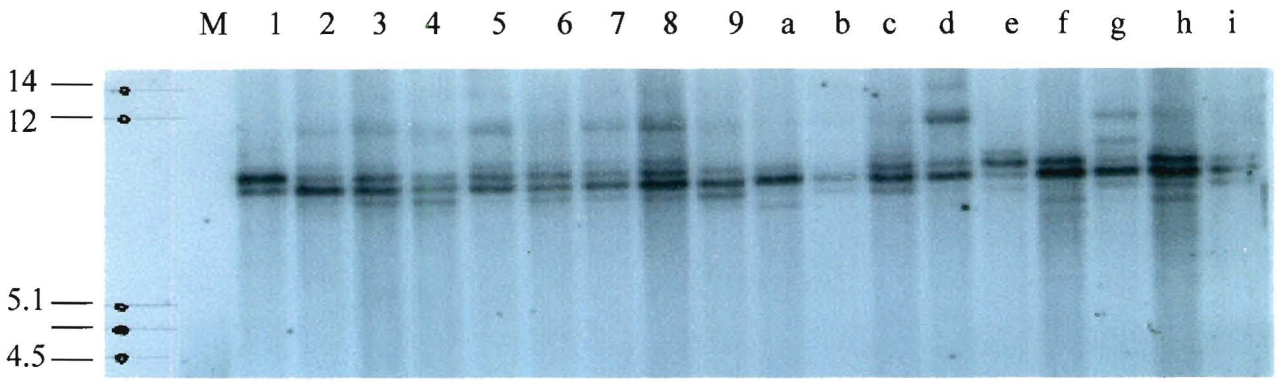

Figure 4.7 The *Dra*I assay. A representative southern blot showing genomic DNA digested with *Dra*I and probed with a DIG-labelled *18S* rDNA probe. Lane M is the molecular weight marker while lanes 1 to 9 represent plants from ambient UV-B (G3A), and lanes a to i are plants from the enhanced UV-B group (G3H). The molecular weight sizes are shown to the left of the figure.

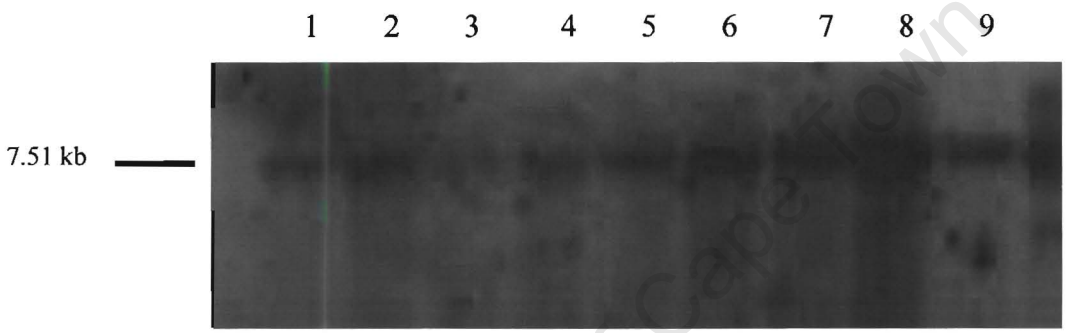

Figure 4.8 The *Dra*I assay. A representative blot of genomic DNA from Generation 0 digested with *Dra*I and probed with a DIG-labelled *rbc*L probe.

4.4 Discussion

Comparison of the nucleotide sequence of the *D. sinuata rbc*L gene with the *D. pluvialis* sequence showed that of the 1458 bp, 26 nucleotides were identified as being different. However, at the protein level, only four of the 486 amino acids were found to be different. Comparison with reported *rbc*L genes from other species show variations in length. For instance the length of the *rbc*L coding region in members of the Asteraceae is 1428, 1431, 1434, or 1458 bp. All these variations occur at the 3' end of the gene and are due to small insertions/deletions and frameshift mutations.

A general characteristic of the 3' untranslated regions of plastid mRNAs is an inverted repeat sequence that can fold into stem-loop structures. These stem loop structures are superficially similar to structures involved in prokaryotic transcription termination, but were found instead to serve as RNA 3'end processing signals in

spinach chloroplasts, and in *atp*B mRNA in *Chlamydomonas reinhardtii* chloroplasts (Rott *et al.,* 1996). The *atp*B gene, which is adjacent and is transcribed divergently to the *rbc*L gene (Hanley-Bowdoin *et al.,* 1985) encodes the beta subunit of chloroplast ATP synthase. The *atp*B and *rbc*L genes have been reported to be located on opposite strands of the maize chloroplast genome. Their transcription start sites are separated by a 159-bp sequence that includes the promoters of both genes (Hanley-Bowdoin and Chua, 1989). The promoters of the two genes have been found to interact in a *cis* and spacing dependent manner and the interaction may have physiological relevance *in vivo.* Inverted repeats sequences found at the 3' end of most chloroplast protein coding regions have been shown to be important for accumulation of *atp*B mRNA in *Chlamydomonas reinhardtii* (Stern and Kindle, 1993). *In vitro* studies indicated that 3'Irs are inefficient transcription termination signals in higher plants and processing activities that act on the 3'ends of chloroplast transcripts have been defined, suggesting that most chloroplast mRNAs are processed at their 3'ends *in vivo.* Since most of the variation in the *rbc*L gene occurs mainly at the 3'end, it would be very interesting to further characterise sequences downstream of the *rbc*L gene in the context of UV-B radiation.

The *Dra*I assay is a technique that is used in assaying genomic DNA for evidence of UV-induced mutations. It was used in this study to analyze variations in the two study populations. The assay is based on the premise that loss of *Dra*I sites would point to evidence for targeted damage by UV. If *Dra*I sites are lost, one would anticipate higher molecular weight bands in plants with an enhanced UV-B exposure history. Both a chloroplast (*rbc*L) and nuclear (*18S* rRNA) probes were used. The significance of evaluating chloroplast as well as a nuclear gene is that mutations in the *rbc*L gene would have been contributed to the seed genetic material through maternal DNA as chloroplasts are maternally inherited (McIntosh *et al.,* 1980).

The *18S* rRNA gene is a single copy gene arranged in tandem repeat. Because of this, intense signals were always obtained, and the probe routinely produced bands ranging both in size and number. This result, however, is contrary to what was proposed in the work by Harlow *et al.* (1994) in which a single band was found when control samples were hybridized with the *18S* rRNA. Harlow *et al.* (1994) attributed the appearance of partial bands to UV-B effects. In this study a number of bands were

found in the control samples that had no known history of UV-B exposure, indicating the presence of variation in the arrangement of the *18S* rRNA tandem repeats of *D. sinuata,* hence the unsuitability of the *18S* rRNA as a probe in the *Dra*I assay.

One well documented result of UV-B is the appearance of double-strand breaks (DSBs) in DNA (Puchta, 1996; Michel *et al.,* 1997) and pyrimidine dimers. The cyclobutane pyrimidine dimer (CPD) (and possibly DSBs) burden resulting from sublethal doses of UV-B may inhibit plant growth and development by slowing transcription and mitosis by imposing energetic costs associated with DNA repair. These DSBs can be repaired via several repair pathways but the most economical is simple ligation with another available DNA strand. This pathway is preponderant in higher eukaryotes but has serious disadvantages since eventually it is accompanied by the loss of genetic material and may even lead to gross chromosomal re-arrangements. The variability observed with the *18S* rRNA probe could have arisen from this.

Basal variation in Generation 0 samples indicated that there was too much variability stemming from the arrangement of the *18S* rRNA gene for conclusive deductions to be made from the study using this assay. Similar levels of variability were observed when control plants (Generation 3A) were compared with test plants (Generation 3H), suggesting that there is inherent variation in the arrangement of the *18S* rRNA gene, and that this was not necessarily a result of stressful growth conditions in the greenhouse. However, the UV-B irradiated samples (G3H), showed slightly greater variability than ambient levels (G3A), even though no bigger bands were evident. This variation could be a result of stress-induced re-arrangement or duplication in the *18S* rRNA gene as opposed to evidence of pyrimidine dimer formation or UV-B induced mutations resulting in loss of *Dra*I sites.

Variation in the number of subrepeat elements per repeat unit probably accounts for much of the differences observed when genomic DNA is probed with the *18S* rRNA. The variability in the *18S* rRNA probably arises from RNA duplications, gene rearrangements and variation in the number of tandem repeats. Only minor variations have been reported in the coding regions of rRNA within a species, but variation is common in the intergenic spacer resulting from a series of repetitive elements in the nontranscribed spacer (NTS) region. Moreover, because rDNA is a repetitive DNA

sequence within the genome, individuals may contain different length variants (Rogers and Bendich, 1987; Agarwal *et al.*, 1992; Amarger and Mercier, 1996). The existence of differences between rDNA repeats of a single genotype in pea with respect to the degree of base modification at certain restriction sites and incomplete cleavage of genomic rDNA have been reported resulting mostly from methylation (Jorgensen *et al.,* 1987). It would be very interesting to look at the effect of UV-B on DNA methylation as this would also help explain the possibility of sequence variation being the cause of observed differences in this study.

The *rbc*L gene is a single copy chloroplast-encoded gene which, like the *18S* rRNA, also gives an intense signal due to the multicopy nature of the chloroplast genome. Since no differences could be detected with this probe, this could be an indication that the actual *rbc*L gene is not mutated or that any original mutations are either lethal or are never seen. Both the 18S ribosomal RNA gene and the *rbc*L gene are present in many thousands of copies in each cell, and therefore a change of less than 5 or 10% may not have been detected. Gene conversion events in the *18S* rRNA gene may have obscured any residual mutations. The *Dra*I assay seems fairly insensitive and is capable of detecting mutations under high doses of UV-B as shown in the work by Harlow *et al.,* (1994), and therefore it becomes inherently difficult to detect residual mutations in the absence of UV-B radiation as evidenced by these results. It is the intention of this author to take this work further by looking at low-copy nuclear encoded genes that are localised in the chloroplast such as the *rbc*S and *chs*. With such genes, the chances to find changes in the nuclear genes may be higher because the frequency to achieve homoplastony is expected to be low.

An alternative approach would be to employ the use of a pyrimidine dimer specific endonuclease to detect pyrimidine dimers directly in DNA. This could however, only be used in situations in which the DNA is irradiated and then immediately assayed for CPDs as was the case in the study by Harlow *et al,* (1994). The other possibility would be to use the RAPDs to identify polymorphisms in the genome. Cullis *et al.* (1999) have studied the occurrence of environmentally induced changes in certain flax varieties and these have been found to be accompanied by changes in the genomic DNA. Such techniques look at changes in the whole genome and are not specific. In this instance, we were looking for evidence of residual mutations at dimer

sites after the plants had been irradiated over several generations and then grown in a greenhouse in the absence of UV-B radiation.

Biochemical and physiological data discussed in the previous chapter point to a facet of the photosynthetic machinery being possibly affected by UV-B radiation. Therefore, if the *rbc*L gene has not been mutated, perhaps the regulation of genes involved in photosynthesis may have been affected. This could explain the biochemical variations observed in plant assays. The next chapter therefore looks at differences in gene regulation in key photosynthetic pathways using northern blot analysis. Biochemical analysis of the same gene products has been carried out to correlate mRNA levels with the actual gene products they code for.

CHAPTER 5

ANALYSIS OF PHOTOSYNTHETIC GENE EXPRESSION AND REGULATION

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Chapter 5

Analysis of photosynthetic gene expression and regulation

5.1. Introduction

Photosynthetic oxygen evolution is catalysed by photosystem II (PSII), a complex of several proteins and pigments located in the chloroplast membrane. This thylakoid membrane protein-pigment complex mediates the initial light-driven reactions in which electrons are removed from water, yielding oxygen and hydrogen ions (Erickson *et al.*, 1989). PSII functions as a light-triggered water-plastoquinone oxidoreductase. The electrons are transferred through a series of intermediate acceptors to the first and second stable quinone acceptors of PSII, Q_A and Q_B respectively.

The reaction-centre core of this photosystem consists of the P680-binding subunits D1 (*psb*A) and D2 (*psb*D), cytochrome b_{559} (*psb*E- *psb*F) and the *psb*I protein, and performs the primary charge separation (Meurer *et al.*, 1998). The minimal oxygen evolving PSII complex in addition contains CP47 (*psb*B) and CP43 (*psb*C), two proteins of the inner chlorophyll *a* antenna, as well as the extrinsic, lumenal 34 kDA (*psb*O). There are approximately 15-20 additional proteins which have been identified as constituent subunits of PSII but their functions have only been partially elucidated. These include bound chlorophylls, pheophytins, quinones, and nonheme iron (Greenberg *et al.*, 1989). PSII, like other thylakoid membrane complexes, is a genetic mosaic and consists of nuclear- and plastome-encoded subunits. The D1 and D2 proteins dimerize to form the photochemical reaction centre core of PSII. The D1/D2 heterodimer binds all of the chlorophylls, quinones, and metal ligands necessary to perform primary PSII photochemistry and electron transport (Lindahl *et al.*, 2000).

The D1 polypeptide is encoded by the chloroplast *ps*bA gene and is an integral part of the active PSII reaction centre core. It has received considerable attention because it is a major product of the chloroplast protein-synthesising machinery and is rapidly turned over as a function of visible light intensity (Aro *et al.*, 1993). D1 is the direct

target for PSII herbicides such as atrazine and diuron. The different herbicides compete with each other and with quinone for binding to the thylakoid membranes. These herbicides block electron transport through PSII and appear to disrupt electron transfer by displacing the bound plastoquinone, Q_B, thus inhibiting D1 protein degradation.

5.1.1. Photoinhibition

Under conditions of high light intensity, electron transport within the PSII reaction centre is arrested, and consequently, the photosynthetic process is inactivated. Excess light can be harmful to the photosynthetic apparatus as it leads to free radical formation and photo-oxidation processes which cause photoinhibition and result in the inactivation of PSII reaction centres, loss of chlorophyll and reduced photosynthetic activity (Andersson *et al.*, 1992; Barber and Andersson, 1992; Andersson and Barber, 1994).). Excessive irradiation results in over-reduction of the electron transfer chain, which eventually causes an impairment of electron transport and inhibition of PSII electron flow and irreversible damage to the subunits of PSII reaction centres accompanied with rapid degradation of D1.

Photoinhibition is a major limitation to photosynthesis under field conditions, and its molecular mechanism has received extensive attention (Greenberg *et al.,* 1989). The main target for light stress is the oxygen-evolving PSII complex in the thylakoid membrane. Among the PSII core proteins, the D1 protein is the main target, although degradation of other PSII components has also been observed (Schroda *et al.*, 1999). Several lines of evidence indicate that distinctly different photosensitizers activate the D1 degradation process in various spectral regions (Greenberg *et al.,* 1989). The data implicate bulk photosynthetic pigments in the visible and far-red regions and quinones in the UV region.

Plants respond to changing light conditions by altered gene expression so that the maintenance of high photosynthetic efficiency is achieved and formation of toxic radicals is minimised under high light fluxes. In other words, plants have evolved several strategies to keep photoinhibition at a minimum. To cope with excess light conditions, plants have developed several mechanisms for adaptation, repair and

protection. Examples include PSII repair via replacement of damaged D1 protein by *de novo* synthesised D1 and protection by quenching of excess excitation energy via the activation of the xanthophyll cycle and light-harvesting complex II (LHCII) phosphorylation.

Plants also minimise photoinhibition through the state transition process, whereby the major antenna protein of the PSII light-harvesting complex becomes phosphorylated and undergoes lateral migration from appressed regions of thylakoid membranes to PSI-rich non-appressed stroma-exposed regions where repair takes place (Baker *et al.,* 1997). The repair of damaged PSII reaction centres is the most important means by which plant cells keep these centres functional during light stress. This antenna movement diverts part of the excess light energy away from PSII and thereby acts as a photoprotection mechanism. In addition, nonradiative dissipation of excess excitation energy can be brought about by de-epoxidation of specific xanthophylls, resulting in non-photochemical quenching of chlorophyll fluorescence (Pfundel *et al.*, 1992; Bornman *et al.,* 1994). Once the capacity of these protective mechanisms has been exceeded, maintaining photosynthetic activity rests with the repair of the photodamaged reaction centres and on the synthesis of new reaction centres. When the rate of photodamage exceeds the rate of repair and replacement, photoinhibition of PSII takes place and the accumulation of damaged PSII centres occurs.

Photoinhibition of PSII induces a series of sequential events leading to the proteolytic degradation of the D1 protein and its replacement by a *de novo*-synthesised protein. The disappearance of D1 during photoinactivation of electron transport has been demonstrated both *in vivo* and *in vitro*. PSII recovery from photoinhibition in most photosynthetic organisms can be regarded as an amplification of a continuous repair process that also occurs in dim light. Thus the molecular machinery required for PSII recovery after photoinhibition is expected to be stimulated rather than specifically induced under light stress conditions.

It is evident that UV-B can potentially impair the performance of all three main component processes of photosynthesis namely : the photophosphorylation reactions of the thylakoid, the CO_2 fixation reactions of the Calvin cycle and stomatal control of CO_2 supply (Allen *et al.,*1998). UV-B radiation could influence carbon reduction by

controlling the diffusion of CO_2 to the site of carboxylation. However, those studies which have examined the impact of UV-B radiation on photosynthesis when the stomatal limitation to CO_2 diffusion is zero *i.e.* at saturating concentrations of CO_2, still demonstrate a reduction in photosynthetic capacity (Middleton and Teramura, 1994). This indicates a direct inhibition of photosynthesis not related to stomatal limitation and that in the absence of direct changes in stomatal conductance, UV-B radiation induces a chronic reduction in photosynthetic capacity.

The changes in photosynthetic capacity may occur at a number of different sites within the photosynthetic carbon reduction cycle. For instance, a number of studies have demonstrated an inverse correlation between increasing UV-B radiation and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity (Strid *et al.*, 1990; Sullivan and Teramura, 1990). Rubisco requires ATP and NADPH produced from the light reactions in order to reduce CO_2 to glucose (see section 4.4). Consequently, it is not surprising to observe reductions in Rubisco activity with supplemental UV-B radiation (Jordan, 1996; A-H Mackerness *et al.*, 1997a). Such reductions could arise because of the decrease in ribulose 1,5-bisphosphate (RuBP) (substrate) regeneration capacity as ATP and NADPH become limited (Strid *et al.*, 1990; Sullivan and Teramura, 1990).

The activities of many components of photosynthesis are regulated by the rates of other photosynthetic reactions. Therefore, examination of a single process or component, such as the carboxylation velocity of Rubisco, the quantum efficiency of PSII or stomatal conductance, does not allow the identification of the primary UV-B limitation (Allen *et al.*, 1998). Numerous investigations have demonstrated that PSII is the most sensitive component of the thylakoid membrane photosynthetic apparatus on exposure to UV-B (Bornman, 1989; Caldwell *et al.*, 1989; Stapleton, 1992; Teramura and Sullivan, 1994; Fiscus and Booker, 1995; Nogues and Baker, 2000). Consequently, PSII damage has often been implicated as the major potential limitation to photosynthesis in UV-B irradiated leaves as is the case in the photoinhibition of photosynthesis by excess photosynthetically active radiation (400 – 700 nm), although it has been suggested that the mechanism of UV-B induced damage may be different (Nogues and Baker, 2000).

The fact that declining levels of stratospheric ozone and the subsequent increase in UV-B radiation may deleteriously affect plant photosynthesis has long been recognised (Teramura, 1996). However there is a wide range of susceptibility in the photosynthetic apparatus with respect to increasing UV-B radiation. Differences in photosynthetic sensitivity to UV-B radiation may be based both upon the biological factors related to leaf optical properties and repair and protective mechanisms as well as physical factors related to experimental conditions, especially the quantity and quality of background radiation. The importance of photosynthetically active radiation (PAR) in determining the magnitude of plant response to UV-B has long been studied (Teramura 1980; Mirecki and Teramura, 1984; Cen and Bornman, 1990). Increased PAR generally decreases the impact of enhanced UV-B radiation on variables such as efficiency and capacity of photosynthesis, leaf area, plant height and pigment concentrations. Despite these reports, some controlled environment studies have continued to use low levels of PAR and, on occasions very high doses of UV-B in the absence of any PAR (Corlett *et al.*, 1997).

The epidermis forms the first effective barrier against the penetration of UV-B radiation into the leaf. In general, the leaf epidermis is very effective in transmitting a large portion of PAR while limiting the amount of UV-B radiation. This effectiveness varies depending upon leaf thickness, the presence of UV-B absorbing compounds and leaf surface properties. Penetration and internal distribution of UV-B radiation varies among plant species, and is strongly affected not only by epidermal thickness but by leaf anatomy, pigments and other physiological changes which result from exposure to UV-B radiation (Teramura *et al.*, 1996). Selective filtering of UV-B radiation in leaves suggests that most plants in their natural environments probably do not suffer reduced photosynthetic capacity as a result of UV-B radiation. However, selective filtration is imperfect as evidenced by the wide range of studies showing photosynthetic inhibition with increased UV-B radiation (Tevini and Teramura, 1989). In addition, it is also possible that despite protective mechanisms, microsites of relatively high levels of UV-B radiation occur because of uneven distribution of screening pigments.

There can potentially be a number of direct and indirect consequences of UV-B radiation on photosynthesis. One of the direct effects is a decrease in the efficiency of

light harvesting reactions, with the D1 protein of the PSII reaction centre being particularly sensitive. In addition, there have been reports that photosynthetic carbon reduction may also be sensitive with UV-B radiation having a direct effect on Rubisco activity and content. Effects on leaf and whole plant development that may indirectly influence photosynthesis including changes in leaf photosynthetic pigments and stomatal conductance have been reported to be indirect results of UV-B radiation. With this in mind, experiments designed to answer these questions were undertaken.

Previous studies indicated many attributes of the photosynthetic system that could be affected by UV-B radiation (Strid *et al.,* 1994; Caldwell *et al.,* 1998). It appeared that the effects of UV-B radiation on photosynthesis, growth and development of plants were caused by altered gene action. This chapter is an analysis of mRNA levels of chloroplast genes directed largely towards photosynthesis since photosynthetic genes and the photosynthetic machinery are most prone to damage by UV-B (A-H Mackerness *et al.*, 1997b, 1998; Jordan, 1998; Strid *et al.,* 1990; Taylor *et al.,* 1996; Surplus *et al.,* 1998). The objective was to carry out an in-depth analysis of mRNA levels of genes involved in photosynthesis and to correlate the mRNA levels of these genes with photosynthetic pigments and secondary metabolites from the same set of plants (see chapter 3). The *Dra*I assay for residual pyrimidine dimers and UV-B-induced mutagenesis did not reveal any evidence of UV-B induced mutagenesis (Chapter 4). However, a lot of basal variation (both in Generation 0 and G3A plants) was found when samples were hybridized with the *18S-rDNA* probe. On average, the test plants had more bands than the control plants, and this variability had been reported to be due to gene re-arrangements arising from stress and this is prevalent in DNA sequences with duplications and tandem repeats. It was postulated therefore that the effect of UV-B could be on systems controlling photosynthetic gene expression, more so that the changes reported in the previous chapters were observed in the absence of additional UV-B radiation.

5.2. Materials and methods

5.2.1. RNA isolation

The same plants that were used for pigment analysis as reported in Chapter 3 were used for mRNA analysis. Leaves of approximately the same age were used to reduce variations due to various stages of development. Approximately one gram of fresh weight leaf material sampled from mid-adaxial positions of six-week-old plants were frozen in liquid nitrogen and stored at –70°C. Total RNA was then isolated using the Trizol Reagent (Gibco BRL – Life Technologies) according to the manufacturer's protocol. Isolations were done once and total RNA was aliquoted into various tubes to minimize variations arising from the stress induced by harvesting leaves at different times. Total RNA was quantified using a spectrophotometer and equal amounts were resolved by electrophoresis on formaldehyde formamide agarose gels to verify quantification. This was done by checking each gel under UV light to ensure both RNA integrity and that equal amounts of RNA were loaded in each lane. Once quantification was completed, total RNA stocks were diluted to 50 ng/µl and aliquoted into different tubes. The samples were stored at –70°C. Each tube was thawed once and used for slot blot analysis.

5.2.2 Northern hybridization

A 48-well slot blot apparatus (Hoefer Scientific Instruments) was used for analysis. Three layers of pre-wetted No.3 Whatman paper were placed onto the slot blot apparatus and a 0.45 µm microporous, positively-charged nylon membrane (Roche Diagnostics GmBH, Mannheim, Germany) that had been cut to the size of the slot blot apparatus was placed over the filter papers. The assembly was tightly clamped together and the apparatus was attached to a vacuum pump. Moisture was removed from the filter paper by applying a vacuum for a minute.

Equal amounts of RNA (500 ng) were then loaded directly onto the nylon in duplicate for each sample and the wells were flushed once with 50 µl of RNAse-free milliQ H_2O to ensure complete loading of RNA onto the membrane. Two membranes were

prepared each time and one was used for the probe of interest, and the other with the internal standard (*18S* rRNA) *i.e.* loading control analysis of each blot was carried out by hybridization using an *18S* rRNA probe (see Surplus *et al.,* 1998). The vacuum was turned on for five minutes, leaving RNA bound onto the nylon membrane. The RNA was then fixed on the membrane using an Amersham UV-Crosslinker (RPN 2500/2501) at a pre-set UV exposure of 70 000 $\mu J/cm^2$ for 10 to 15 seconds. The cross-linked membrane was incubated in 2X SSC for 2 minutes, and then placed in a hybridization bag containing 20 ml pre-warmed Eazy Hyb solution (Roche Diagnostics GmBH, Mannheim, Germany) per 100 cm^2 of membrane surface. The bag was sealed and then prehybridized at 42°C for 30 minutes.

Preparation of probes and hybridization

The *psb*A gene probe was supplied by S. A-H Mackerness as an 850-bp fragment containing the 3' 60% of the gene from spinach cloned into the *Hind*III site of the plasmid pBR322, selected on ampicillin resistance (A-H Mackerness *et al.,* 1997b). The probe was made by restriction endonuclease digestion of the construct with *Hind*III to release the insert, which was excised out of a normal 0.8% agarose gel and purified with the Roche Biochemicals High Pure PCR product purification kit according to the manufacturer's protocol. The purified insert was then Digoxigenin-(DIG) labelled using the random-primed labelling method. The *18S* rRNA and *rbc*L probes were prepared as described in chapter 4, (sections 4.2.2.1 and 4.2.2.2, respectively). The DNA probe was incubated in a boiling water bath for 10 minutes to denature the DNA and then placed immediately on ice. The prehybridization solution was poured off and the hybridization solution containing the DIG-labelled probe was added at a concentration of 25 ng/ml and hybridization was carried out for 12 hours at 42°C. After hybridization, the membrane was washed twice for 5 minutes in 2X SSC + 0.1% SDS at room temperature, followed by two 15-minute washes in 0.1X SSC + 0.1% SDS at 65°C.

Detection of DIG-labelled nucleic acids.

Following hybridization, signals were detected using the CSPD chemiluminescent alkaline phosphatase substrate according to the supplier's protocol (Roche

Diagnostics GmBH, Mannheim, Germany). The membrane was equilibrated for 2 minutes in Buffer 1 (100 mM maleic acid, 150 mM NaCl; pH 7.5), then blocked in blocking solution (1% skim milk powder in Buffer 1) for 30 minutes. The membrane was incubated in anti-DIG solution (Anti-Digoxigenin–AP diluted 1:10 000 in blocking solution) for 30 minutes following which it was washed twice for 15 minutes in wash buffer (Buffer 1 + 0.3% Tween 20®) at room temperature. The membrane was then equilibrated in detection buffer (Buffer 3 – 100 mM Tris-HCl, 100 mM NaCl; pH 9.5) for 2 minutes, after which it was incubated in CSPD solution (CSPD diluted 1:100 in detection buffer) for 5 minutes. The CSPD substrate was allowed to reach steady state by incubating at 37°C for 10 minutes. The CSPD solution was retained for further use. The membrane was placed in a sealed hybridization bag, placed in an X-ray cassette, and exposed to X-ray film. The blot was developed after an appropriate exposure time, which was determined empirically. All reagents for detection of DIG were supplied by Roche Diagnostics GmBH Mannheim, Germany, unless otherwise stated.

Hybridization with the different probes (*rbc*L, *psb*A and *18S* rRNA) was done in separate bags. Washes and detection were carried out in the same bag with the same solutions. One membrane was probed with either the *rbc*L or *psb*A probes and the other was hybridized with an *18S* rRNA probe. Membranes were exposed on the same X-ray film for the same duration. After appropriate incubation, blots were developed and the intensities of the resulting bands were determined using the Macbeth Transmission Densitometer (TD-901). Readings were taken for each slot on the membrane for all the samples, and the corresponding densitometer reading for the same plant was determined for the *18S* RNA. After all the values were compiled, ANOVA (see section on statistical analysis) was carried out, and variations in loading were accounted for in the analysis by using the *rbc*L/*18S* rRNA and psbA/*18S* rRNA ratios *i.e.* the *18S* rRNA gene expression was used as an internal standard to normalize the results. The ratios R_r and R_p were defined as R_r = *rbc*L/*18S* rRNA and R_p = *psb*A/*18S* rRNA.

5.2.3. Statistical analysis

The ratios of the observations of each probe (*rbc*L and *psb*A) to their respective *18S* rRNA readings were used in the analysis. A nested mixed effects analysis of variance was used to test the differences between the radiated and control plants and to estimate the variation due to plant, blots and duplicate observations on the blot *i.e.* the relative contribution by each factor was investigated (Scheffe, 1959; Hicks, 1982). Missing values present on any of the blots were estimated using an iterative missing value formula, which ensured that the missing values did not contribute to the variation.

Let y denote a single ratio reading identified by the subscripts ijkl which represents treatments, plants, blots and duplicates, then: $y_{ijkl} = \mu + \alpha_i + b_{j(i)} + c_{k(ji)} + d_{l(kji)}$ where b, c, d are random variables representing variation due to plants, blots and duplicate readings on the blots respectively. Subscripts within brackets denote the nesting. The differences between the irradiated and control plants were assessed using the variation due to plants. The variation due to blots and duplicates measures the technical (*i.e.* measurement) errors. These sources were estimated and compared both separately in the radiated and control plants and overall. The Genstat statistical package (Payne, 1998) was used for the computations.

5.3. Results

5.3.1 Determination of mRNA levels

To determine mRNA levels for the *rbc*L, *psb*A and *18S* rRNA genes, blots were hybridized independently with the corresponding probe for at least 12 hours and the densitometer readings were determined. Figure 5.1 is a representative blot of control and test plants probed with *psb*A and *18S* rRNA probes. The corresponding densitometer readings from these blots (Figure 5.1) are presented in Table 5.1.

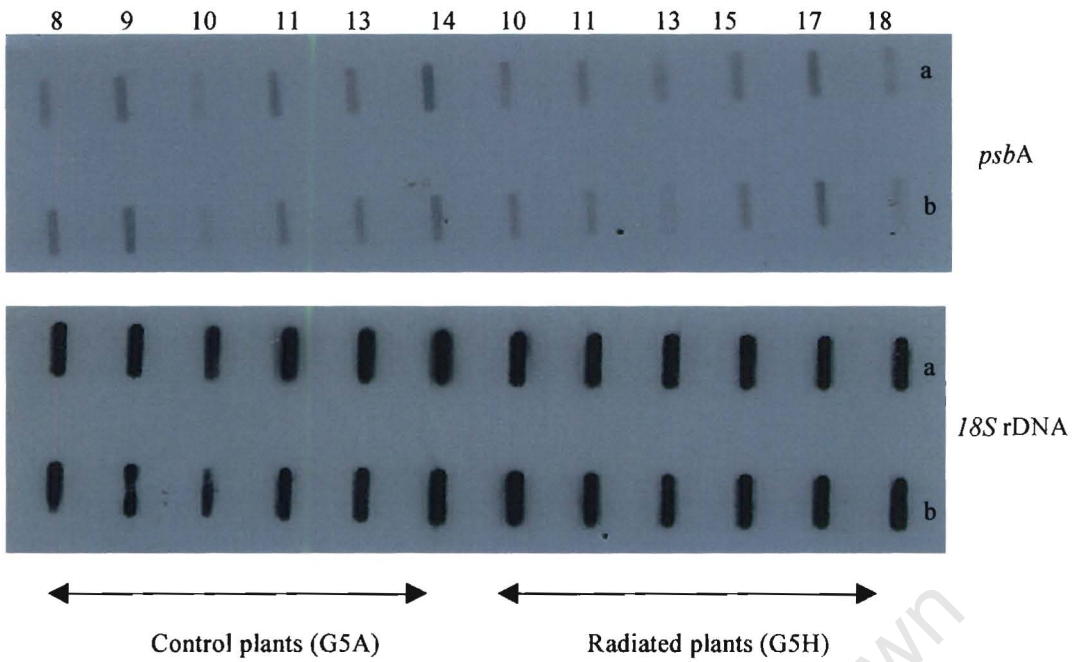


Figure 5.1. Representative blot of both control and test plants probed with an *18S* rDNA probe (lower panel) and a *psb*A probe (upper panel). Both membranes were exposed for the same duration on the same X-ray film to eliminate errors arising from exposure time. G5A and G5H represent fifth generation control and radiated plants, respectively, grown from seeds taken from the fourth generation. The numbers at the top of the blot represent arbitrary numbers allocated to individual plants. The letters *a* and *b* represent duplicate readings for each probe (*a* = Reading 1, and *b* = Reading 2).

Table 5.1. Example of calculation of densitometer readings taken from the blot in Figure 5.1 for both *psb*A and *18S* rRNA probes and the corresponding *psb*A: *18S* rRNA ratios.

	***psb*A readings**			***18S* rRNA readings**			**Ratio**
Sample	Reading 1	Reading 2	*psb*A	Reading 1	Reading 2	*18S* rRNA	*psbA/18S*
C*8	0.31	0.34	0.325	1.11	1.11	1.110	0.292793
C9	0.35	0.37	0.360	1.05	1.03	1.040	0.346154
C10	0.27	0.27	0.270	0.78	0.68	0.730	0.369863
C11	0.32	0.32	0.320	1.06	0.95	1.005	0.318408
C13	0.32	0.32	0.320	1.05	0.93	0.990	0.323232
C14	0.39	0.34	0.365	1.20	1.16	1.180	0.309322
T*10	0.30	0.31	0.305	1.24	1.22	1.230	0.247967
T11	0.29	0.29	0.290	1.16	1.14	1.150	0.252174
T13	0.30	0.26	0.280	1.02	0.91	0.965	0.290155
T15	0.31	0.31	0.310	1.15	1.12	1.135	0.273128
T17	0.35	0.34	0.345	1.13	1.15	1.140	0.302632
T18	0.28	0.28	0.280	1.14	1.12	1.130	0.247788

* (C and T before the sample number represent control and treated (radiated) plants, respectively).

rbcL mRNA levels and rbcL:18S rRNA *ratios*

A total of twenty-one control plants and twenty-three radiated plants were analysed with the *rbc*L probe. Table 5.2 represents mean values for the *rbc*L mRNA levels, and the corresponding standardized ratios computed from the *18S* rRNA internal standard. Except for plant T9 which had two observations, at least four observations were made for each plant, from a total of ten blots. The mean values are represented graphically in Figure 5.2. The overall mean ratios for the control and radiated plants were found to be 0.703±0.050 and 0.669±0.067 respectively (Figure 5.2), *i.e.* treated plants were lower than the control plants.

psbA mRNA levels and psbA:18S rRNA *ratios*

A total of twenty-one control plants and twenty-five radiated plants were analysed with the *psb*A probe (Table 5.3). Table 5.3 represents mean values for the *psb*A mRNA levels, and the corresponding standardized ratios computed from the *18S* rRNA internal standard. Except for plants T5, T8 and T9 which had two observations each (Table 5.3), at least four observations were made for each plant, from a total of eleven blots. The mean values are represented graphically in Figure 5.3. The overall mean *psb*A ratios for the control and radiated plants were found to be 1.2346±0.149 and 1.1618±0.148 respectively, *i.e.* treated plants were lower than the control plants.

Table 5.2. Mean *rbc*L and *18S* rRNA mRNA levels and their ratios from densitometer readings. The values represent means of at least four independent observations.

Control plants					Radiated plants				
Plant No	**N[a]**	**Mean rbcL**	**Mean *18S***	**Mean ratio**	**Plant No**	**N**	**Mean *rbc*L**	**Mean *18S***	**Mean ratio**
C*1	4	1.3850	1.1950	1.1959	**T*1**	4	0.6575	0.9925	0.6774
C2	8	0.5903	0.9823	0.6545	**T2**	4	0.5100	1.1800	0.4379
C3	4	1.7950	1.5800	1.1511	**T3**	4	0.9025	1.1175	0.8258
C4	8	0.7144	0.9413	0.7461	**T4**	4	1.6275	1.1925	1.4101
C5	4	1.1125	1.1650	0.9756	**T5**	4	0.6775	1.0700	0.6514
C6	4	0.2763	0.5450	0.5347	**T9**	2	0.5435	0.3050	1.7768
C8	7	0.7956	1.6370	0.7035	**T10**	8	0.8120	1.7280	0.6972
C9	8	1.1240	1.8910	0.9432	**T11**	8	0.6520	1.4450	0.7342
C10	7	0.4450	1.5289	0.4523	**T12**	4	0.1950	0.4178	0.5013
C11	7	0.8720	1.7222	0.7324	**T13**	7	0.8700	1.7100	0.6869
C12	4	0.2248	0.2865	0.8163	**T14**	4	0.1685	0.5483	0.2918
C13	8	0.7180	1.7300	0.6185	**T15**	8	0.7080	1.7100	0.6800
C14	8	1.0710	1.8950	0.9191	**T16**	4	0.2925	0.6423	0.4776
C15	4	0.4213	0.5568	0.8071	**T17**	8	0.3840	1.4140	0.4736
C16	4	0.2633	0.5828	0.4646	**T18**	8	0.7400	1.6840	0.6835
C21	4	1.1575	2.5350	0.4564	**T19**	6	0.3353	0.4632	0.6846
C22	4	1.3800	2.4900	0.5542	**T20**	4	0.4930	0.6370	0.7260
C23	4	1.3050	2.4425	0.5353	**T21**	4	1.2000	2.4575	0.4904
C24	4	1.2325	2.3975	0.5140	**T22**	4	1.3800	2.4975	0.5531
C25	4	1.2075	2.4950	0.4838	**T23**	4	1.2075	2.4750	0.4870
C26	4	1.2475	2.4725	0.5058	**T24**	4	1.1000	2.4025	0.4577
					T25	4	1.1600	2.4225	0.4790
					T26	4	1.1775	2.3550	0.5010

N[a] = number of observations per plant. * (C and T before the sample number represent control and treated (radiated) plants, respectively).

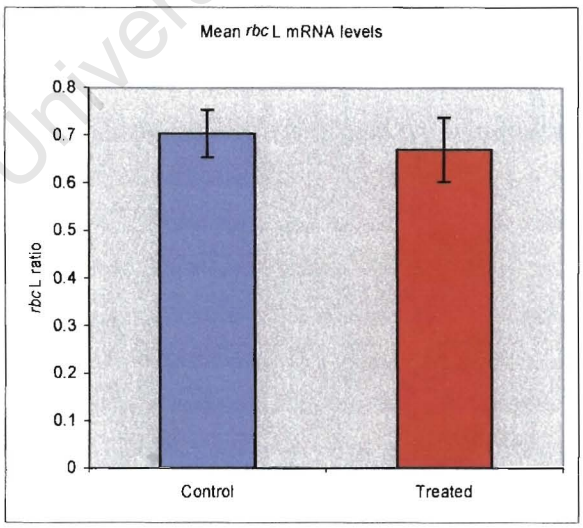


Figure 5.2. Histogram showing mean *rbc*L:*18S* rRNA ratios. Control = 0.703±0.050, treated = 0.669±0.0676. The error bars represent the standard error.

Table 5.3. *psb*A mRNA and *18S* rRNA mRNA levels and the corresponding ratios from densitometer readings.

Control plants					Radiated plants				
Plant No	**N[a]**	**Mean *psb*A**	**Mean *18S***	**Mean ratio**	**Plant No**	**N**	**Mean *psb*A**	**Mean *18S***	**Mean ratio**
C*1	4	0.2933	0.6118	0.5809	**T*1**	6	0.2122	0.472	0.4442
C2	10	0.2221	0.4091	0.8057	**T2**	6	0.3658	0.630	0.6378
C3	6	0.5275	0.5323	0.6347	**T3**	4	0.4630	0.500	0.9386
C4	10	0.3797	0.4069	1.1166	**T4**	6	0.3798	0.637	0.6166
C5	6	0.3667	0.5167	0.7577	**T5**	2	0.4500	0.775	0.5808
C6	10	0.3014	0.4187	0.7011	**T7**	4	0.3071	0.236	1.0418
C8	4	0.2805	0.6193	1.0650	**T8**	2	0.2100	0.323	0.6624
C9	4	0.3448	0.5933	1.3073	**T9**	2	0.1105	0.162	0.6826
C10	4	0.1583	0.4283	0.3699	**T10**	4	0.2398	0.726	0.5191
C11	8	0.3894	0.5904	0.8396	**T11**	4	0.1768	0.638	0.3817
C12	8	0.2795	0.1984	1.4849	**T12**	8	0.1373	0.289	1.7687
C13	4	0.4433	0.6495	1.0791	**T13**	6	0.1962	0.480	0.5591
C14	4	0.4620	0.8003	0.8209	**T14**	8	0.1638	0.141	1.3497
C15	6	0.2833	0.4155	0.6333	**T15**	6	0.2368	0.508	0.7901
C16	4	0.2170	0.2720	1.1967	**T16**	6	0.2425	0.259	0.9566
C21	6	0.3195	0.2460	1.2950	**T17**	4	0.3750	0.740	0.7488
C22	4	0.5788	0.2388	2.4319	**T18**	6	0.2428	0.572	0.7329
C23	4	0.3138	0.2400	1.3159	**T19**	10	0.3001	0.397	1.0659
C24	4	0.4243	0.1818	2.4450	**T20**	4	0.3678	0.247	1.5110
C25	4	0.4170	0.1873	2.3209	**T21**	4	0.4775	0.189	2.6957
C26	4	0.4853	0.1875	2.7242	**T22**	4	0.3570	0.190	1.9400
					T23	4	0.2415	0.185	1.3001
					T24	4	0.4058	0.195	2.1110
					T25	4	0.4308	0.196	2.2802
					T26	4	0.5780	0.215	2.7311

N[a] = number of observations per plant. * (C and T before the sample number represent control and treated (radiated) plants, respectively).

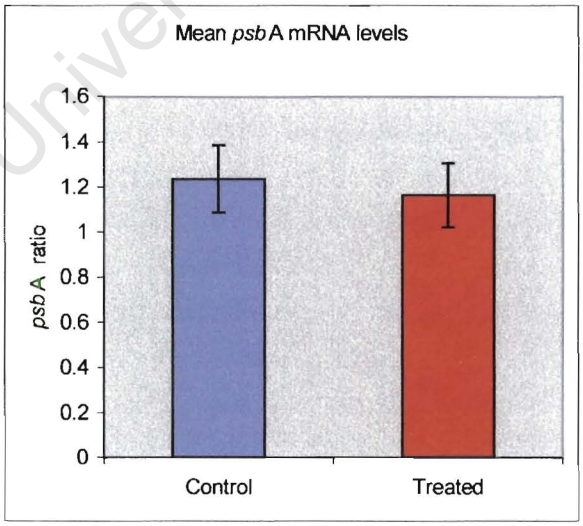


Figure 5.3. Histogram showing the mean *psb*A:*18S* rRNA ratios. Control = 1.2346±0.149, treated = 1.1618±0.148. The error bars represent the standard error.

5.3.2 Statistical analysis

A difference was observed in the mean ratios for mRNA levels of the two genes in which radiated plants were on the whole lower than control plants. However there was no statistically significant difference between the mean mRNA levels from irradiated and control plants for either gene ($F = 1.28$; $df = 42$; $p = 0.264$) and ($F = 0.29$; $df = 44$; $p = 0.593$).

Analysis of sources of variation

A number of factors contributed to the variation observed in the measurements and the relative contribution by each factor was investigated *i.e.* differences in the variance of the radiated and control plant mRNA levels were investigated (Tables 5.4 and 5.5).

*Analysis of sources of variation for the rbc*L *probe*

The estimated stratum variances for the *rbc*L mRNA levels are indicated in Table 5.4.

Table 5.4. Estimated stratum variances for the *rbc*L ratio (R_r). The percentage column gives the percentage of the total variation that can be attributed to each source. d.f = degrees of freedom and rep = duplicate samples.

	Control plants			**Radiated Plants**		
Stratum	Variance	Effective d.f.	Percentage	Variance	Effective d.f.	Percentage
Plant	0.2566	20	36.6	0.30606	22	49.3
Blot	0.4216	37	60.0	0.30131	34	48.5
Rep	0.0212	55	3.00	0.0693	56	1.00

Approximately 50% of the total observed variation in the irradiated plants was due to plants, and the other 50% was due to the blots. For the control plants, 60% of the observed variation was due to blots and 37% was due to plants. The variation due to duplicates was 3% and 1% for the control and radiated plants respectively (Table 5.4).

The stratum variances (error due to each parameter e.g. plant, blot and replicates) of the radiated and control ratios were computed separately and compared with an F-test as shown below.

Source	df	Variance	Fstat.	p-value
Radiated	22	0.3061	1.19	0.3499
Control	20	0.2566		

There was no significant difference in the variation between the control and treated plant standardized *rbc*L mRNA levels ($F = 1,19$, $p = 0.35$).

Analysis of sources of variation for the psbA probe

The estimated stratum variances for the *psb*A mRNA levels are shown in Table 5.5.

Table 5.5. Estimated stratum variances for the *psb*A ratio (R_p). The percentage column gives the percentage of the total variation that can be attributed to each source. d.f = degrees of freedom and rep = duplicate samples.

	Control plants			Radiated Plants		
Stratum	Variance	Effective d.f.	Percentage	Variance	Effective d.f.	Percentage
Plant	2.0325	20	63.9	1.98933	24	77.5
Blot	1.1048	38	34.8	0.55738	35	21.7
Rep	0.0410	59	1.30	0.01368	60	0.5

For the control plants 64% of the total variation was due to plants and 35% of the variation was due to blots. For the irradiated plants, 78% of the total variation was due to plants, while only 22% came from the blots. Only 1.3% and 0.5% of the total variation was caused by errors in the duplicates for the control and irradiated plants, respectively. A comparison of the variances of radiated and control plants was carried out using an F-test and the results are indicated below.

Source	d.f	Variance	Fstat.	p-value
Radiated	24	1.98933	1.022	0.4748
Control	20	2.0325		

There was no significant difference in the variation between the control and treated plant *psb*A mRNA levels ($F = 1.022$; $p = 0.4748$).

5.3.3. Correlations between photosynthetic pigments and mRNA levels for photosynthetic genes.

The different photosynthetic pigments that were measured as described in Chapter 3 were correlated with mRNA levels for the two photosynthetic genes (*psb*A and *rbc*L). The UNISTAT® Statistical Package Version 4.53 (UNISTAT Ltd, 1984-1997) was used to test for significance in the correlations. The results are shown in Table 5.6 and Figures 5.4, 5.5 and 5.6. The ratio for each respective gene (*rbc*L or *psb*A) to the corresponding *18S* rRNA value was used in the correlations.

Table 5.6. Test for correlations between the *rbc*L and *psb*A mRNA levels and photosynthetic pigments. Plants from an ambient UV-B history are represented in the top half of the table while plants from an elevated UV-B history are shown in the lower half of the table. Significant correlations are shown in **bold** print.

CONTROL PLANTS	**Chlorophyll *a***		**Chlorophyll *b***		**Total Chlorophyll**		**Carotenoids**		**Chlorophyll *a/b***	
	R[a]	**T[b]**	**R**	**T**	**R**	**T**	**R**	**T**	**R**	**T**
rbcL/18S	0.4249	2.0460	0.2520	1.1349	0.3884	1.8371	0.4794	2.3832	0.2074	0.9241
psbA/18S	-0.4127	-1.9751	-0.3152	-1.4477	-0.3956	-1.8774	-0.7717	-5.2890	-0.0385	-0.1681
TREATED PLANTS	**Chlorophyll *a***		**Chlorophyll *b***		**Total Chlorophyll**		**Carotenoids**		**Chlorophyll *a/b***	
	R	**T**	**R**	**T**	**R**	**T**	**R**	**T**	**R**	**T**
rbcL/18S	0.2531	1.1989	0.2011	0.9407	0.2416	1.1408	0.3016	1.4497	0.1136	0.5238
psbA/18S	-0.7523	**-5.4771**	-0.7537	**-5.4992**	-0.7607	**-5.6196**	-0.7673	**-5.7385**	0.2394	1.1828

R[a] represents the regression index and T[b] is the t-test value that was used to test for significance in the correlations.

Table 5.6 shows a significant negative correlation between the mRNA levels of the *psb*A gene and chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids. This is clearly depicted in Figures 5.4 to 5.6. Correlations between mRNA levels of the *psb*A gene and all the photosynthetic pigments were found to be highly significant (less than $p = 0.001$) except for the except for the chlorophyll *a/b* ratio. No significant

correlations were found between the mRNA levels of the *rbc*L gene and the photosynthetic and secondary pigments.

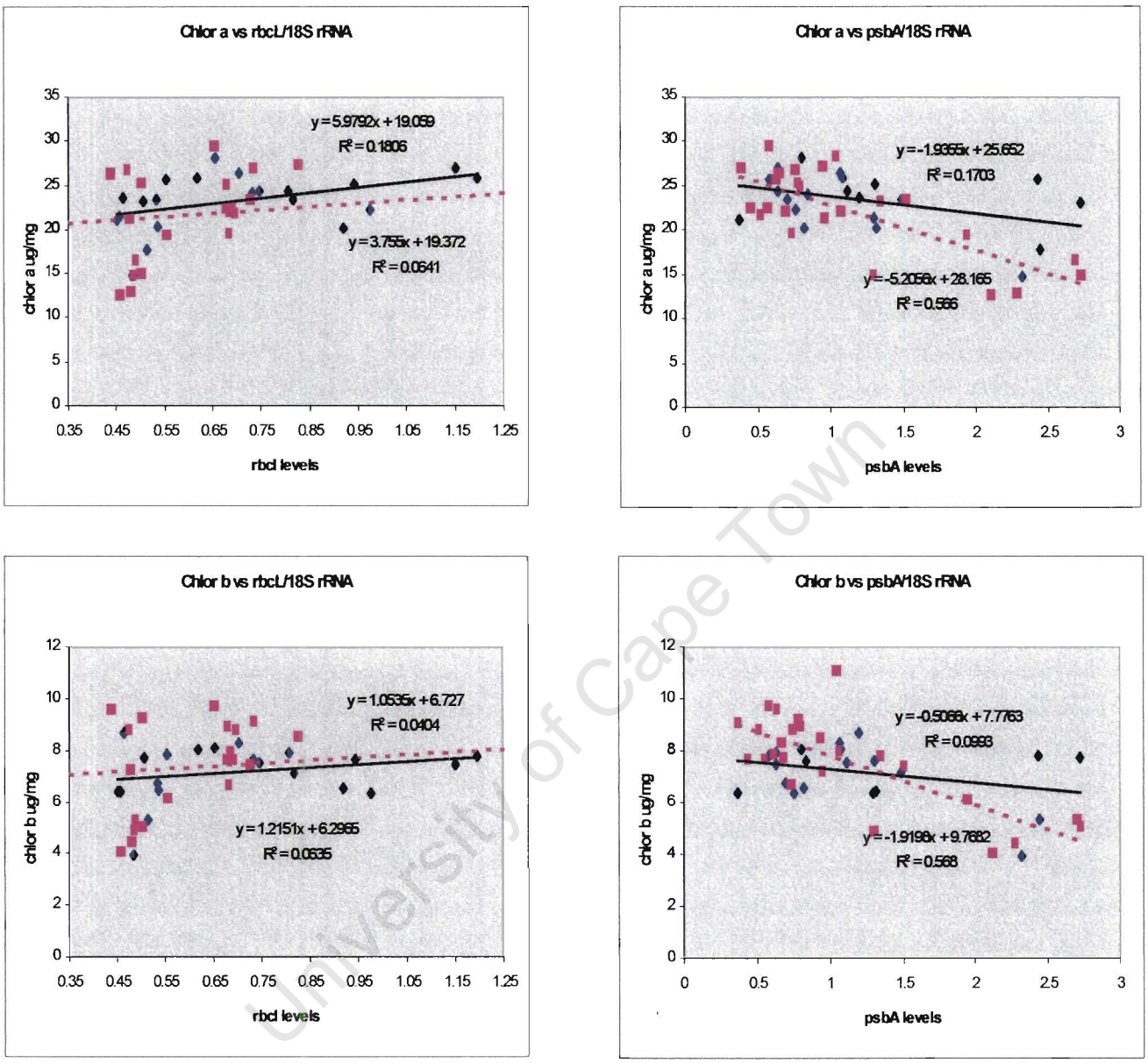


Figure 5.4. Relation between the photosynthetic genes (*rbc*L and *psb*A) and photosynthetic pigments (chlorophyll *a*, and *b*). The solid line represents plants from ancestors with an ambient UV-B history, while the dotted line represents plants from ancestors with an enhanced UV-B history. The regression index (R^2) is shown for each graph. The shaded diamonds represent values from controls, and the shaded rectangles represent values from test plants. Each point represents a mean of at least three independent observations. Chlor a and Chlor b refer to chlorophyll *a* and chlorophyll *b* respectively; 18S rRNA, psbA and rbcl refer to the mRNA for the *18S* rDNA, *psb*A and *rbc*L genes respectively.

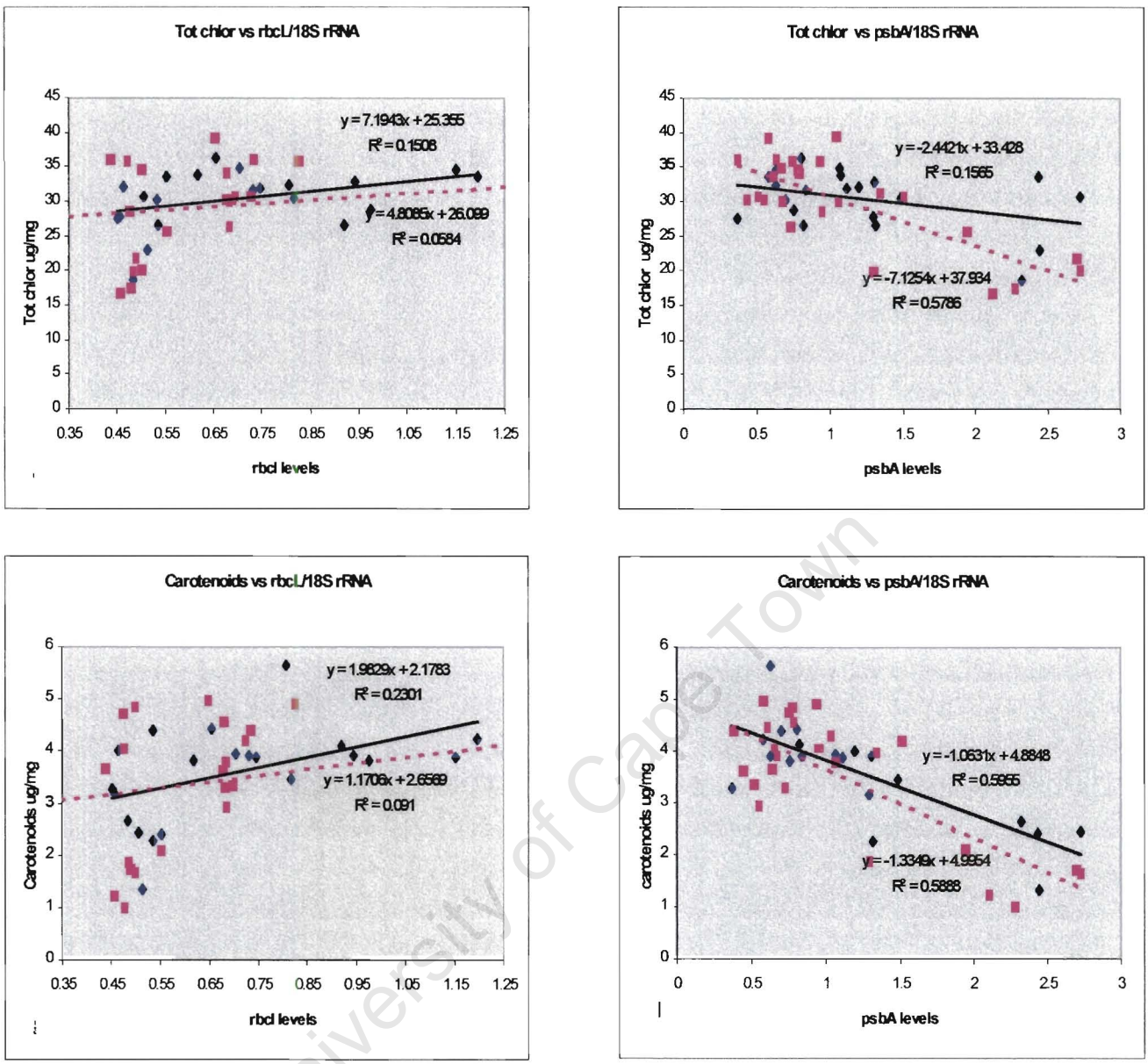
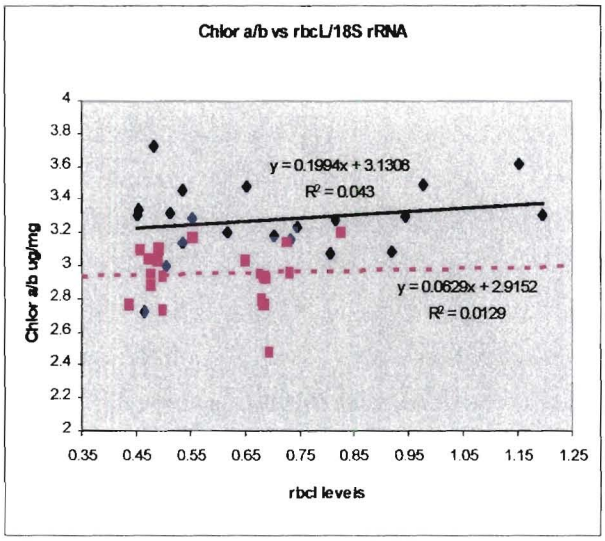


Figure 5.5. Relation between the photosynthetic genes (*rbc*L and *psb*A) and photosynthetic pigments (total chlorophyll and carotenoids). The solid line represents plants from ancestors with an ambient UV-B history, while the dotted line represents plants from ancestors with an enhanced UV-B history. The regression index (R^2) is shown for each graph. The shaded diamonds represent values from controls, and the shaded rectangles represent values from test plants. Each point represents a mean of at least three independent observations. Tot chlor refers to total chlorophyll; 18S rRNA, psbA and rbcl refer to the mRNA for the *18S* rDNA, *psb*A and *rbc*L genes respectively.



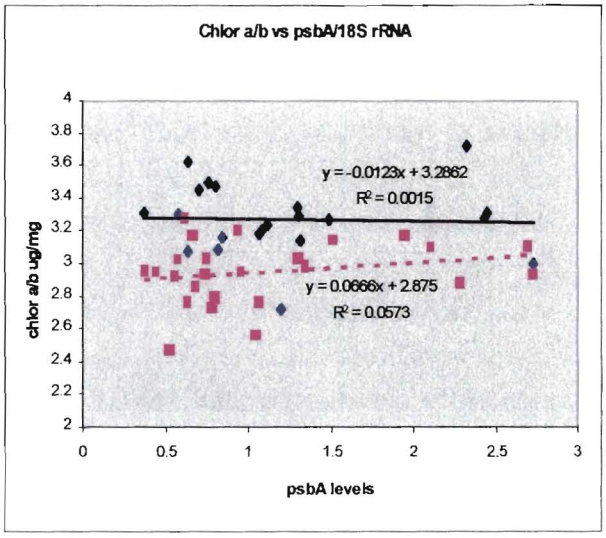


Figure 5.6. Relation between the photosynthetic genes (*rbc*L and *psb*A) and chlorophyll (*a*/*b*) ratio. The solid line represents plants from ancestors with an ambient UV-B history, while the dotted line represents plants from ancestors with an enhanced UV-B history. The regression index (R^2) is shown for each graph. The shaded diamonds represent values from controls, and the shaded rectangles represent values from test plants. Each point represents a mean of at least three independent observations. Chlor a/b is the chlorophyll *a*/*b* ratio; 18S rRNA, psbA and rbcl refer to the mRNA for the *18S* rDNA, *psb*A and *rbc*L genes respectively.

5.4. Discussion

The results from the experiments that were conducted during the earlier stages of this study using plants from generations one to four showed reductions in photosynthetic activity, biomass and several other physiological parameters (Musil, 1996). It was hypothesised that the observed changes could possibly be a result of DNA damage. This was tested using the *Dra*I assay (Chapter 4) but no direct evidence for UV-B-induced mutations was found. It was proposed that a change in the actual genes or in regulation of genes involved in photosynthesis could have occurred at some stage in the history of the plants. The *rbc*L and *psb*A genes were chosen for analysis and these were used as probes to determine the expression of the respective mRNA levels. Tests for correlations between the expression of mRNA levels of the two genes and photosynthetic pigments from the same plants (Chapter 3) were carried out.

The results for the *rbc*L and *psb*A gene probes (Figures 5.2 and 5.3) showed that on the whole there was a reduction in the expression of mRNA levels for both genes.

These results are in agreement with results from previous studies done on the set of same genes using supplementary UV-B radiation for a short duration (Jordan *et al.*, 1991, 1992; A-H Mackerness *et al.*, 1997b; Surplus *et al.*, 1998 and A-H Mackerness, 2000). Although the pattern was consistent there was no statistically significant difference between the mRNA levels for either gene in the irradiated and control groups ($F = 1.28$; $df = 42$; $p = 0.264$ for *rbc*L and $F = 0.29$; $df = 44$; $p = 0.593$ for *psb*A). A total of twenty-one control plants and twenty-three irradiated plants were used in the *rbc*L analysis. For the *psb*A gene probe, twenty-one control and twenty-five irradiated plants were used. More samples need to be analysed in future in order for the statistical analyses to be more rigorous.

Comparison of the variances of irradiated and control plant *rbc*L mRNA levels were done and it was found that most of the variation was contributed by the blots for control plants (experimental error) whereas the contribution by the plants and the blots were almost the same for the radiated plants (Table 5.4). However, when the variation in the control and radiated plants was compared with an F-test, there was no difference in the variation in the control and treated plant *rbc*L mRNA levels ($F = 1.19$, $p = 0.35$). One major limitation of this technique comes from the probe concentration. There is an almost linear relationship between probe concentration and the number of target sites that the probe binds to. As a result, probe concentration might reach saturation earlier for certain samples. What these means is that the probe concentration must be standardised/uniform, but it becomes problematic when one has to assay large samples and has to reuse the same probe more than once. To investigate this, data from blots that contained the same plants that were replicated at least five times (representing a true replication of the experimental technique) were used. Despite the fact that differences were observed in the mRNA levels from the same plants on different blots, the variation was not statistically significant.

Determining the linear range for a given probe, then working with probe concentrations and exposure time within that range could authenticate this observation in future. This was not done in this study as the use of the *18S* rRNA probe as internal standard has been reported to serve the same purpose in previous studies (Surplus *et al.*, 1998). In addition analysis of differences observed from repeated measures were statistically insignificant. However, a number of different strategies

can be applied if it is desired to do a single exposure. Possibilities are to reduce the specific activity of the *18S* rRNA probe by reducing the level of labelled nucleotide in the labelling mix, diluting the labelled probe with unlabelled probe, or reducing the amount of labelled probe in the hybridization reaction. However, the last strategy introduces an additional problem in terms of being sure that the label bound is a true reflection of the homologous RNA on the membrane. It should be noted that the initial rate of reaction in the hybridization is dependent on the concentration of probe, and since there is a great deal of *18S* rRNA on the filters, the probe will be diluted rapidly and the reaction may not got to completion. To get around this problem, a time course of hybridization giving the initial rates of reaction could be carried out in future.

An analysis of variance for the *psb*A probe showed that most of the observed variance was caused by differences in the expression of mRNA levels in the actual plants used and a smaller percentage was due to experimental error *i.e.* due to the blots. A test of significance in the observed values between irradiated and control plants using the F-test showed no significant difference between the variances in the two plant groups ($F= 1.022$, $p = 0.47$). It should be noted that the *psb*A analyses were carried out after the *rbc*L experiments when the technique had improved considerably.

There was no correlation between the *rbc*L mRNA levels and photosynthetic pigments. This is not surprising given the role of the *rbc*L gene in photosynthesis is mainly in carbon fixation which is not related to the pigments studied, hence the lack of correlation. The reduction in *rbc*L levels could however be linked to results from a previous study (Musil *et al.,* 1999) in which a reduced photosynthetic rate was found in plants from the same seed set as the one used in this study. These observation is in agreement with recent studies in which it was shown that under realistic UV-B conditions,, reduction in RUBISCO levels is the primary cause of the decline in photosynthetic rate (Allen *et al.,* 1997; Baker *wt al.,* 1997). Changes in *rbc*L could therefore be related to effects on photosynthesis as evidenced by effects on carbon fixation. In addition, changes in carbon fixation can be attributed to the observed decline in biomass but it should be noted that this could be due to effects on PSII rather than directly on carbon fixation.

There was however, a negative correlation between *psb*A mRNA levels and photosynthetic pigments. The *psb*A gene encodes the D1 protein that is involved in electron transport. Accessory pigment molecules play a crucial role in light capture and photoinhibition during photosynthesis. Could there be a compensatory mechanism at play in the test plants such that at low *psb*A levels one observes higher levels of pigments? Possibly, at high *psb*A levels, there is not as much need for accessory pigments as electron transport is most likely proceeding at an efficient rate. The *psb*A gene product, *i.e.* the D1 protein turnover would be sufficient to allow for photosynthetic capacity to proceed at a more or less normal rate for adequate physiological functioning of the plant.

The D1 protein is encoded in the chloroplast genome by the *psb*A gene, and its synthesis in the chloroplast is regulated by nuclear encoded factors that are synthesised in the cytoplasm and imported into the chloroplast. Degradation of the D1 proteins is a consequence of photo-inactivation of PSII and is generally considered to result from protease activity. Consequently, repair of photodamaged PSII complexes requires the synthesis of D1 in the stroma and insertion and integration of the newly synthesised proteins into the damaged PSII complexes. Given that supplementary UV-B radiation may decrease the activity and content of the PSII complex with a resulting decrease in electron transport, and presumably ATP synthesis, there could be corresponding decreases in photosynthetic capacity and maximum quantum yield (Strid *et al.,* 1990). UV-B radiation may affect photosynthesis indirectly by photobleaching and photodegradation of photosynthetic pigments. High levels of UV-B radiation in combination with low levels of PAR have been reported to significantly reduce chlorophyll content in bean, barley, corn, pea and soybean (Trebst and Depka, 1997). There has been an erroneous tendency to directly equate impaired accumulation of pigment to its impaired synthesis. Preferential reduction of chlorophyll *b* accumulation may be a secondary consequence of the higher affinity of reaction centre apoproteins for chlorophyll *a*, possibly depleting chloroplasts of chlorophyll *b* synthesis.

Carotenoids absorb light of approximately 400 – 495 nm. Some of the light energy absorbed by β-carotene and lutein (up to 40%) can be transformed to chlorophyll

although these carotenoids also help protect against photooxidative damage. Two major functions of carotenoids in photosynthesis are light harvesting and photoprotection of reaction centres, pigment protein antennae, and cells and tissues. Zeaxnathin and antheraxanthin formed from violaxanthin by the xanthophyll cycle increase non-radiative dissipation of energy as heat in the pigment bed of the antenna of PSII. Photoprotection involves removal of singlet oxygen by carotenoids and the formation of triplet states of carotenes. β-carotene molecules have been reported to function in protecting the reaction centre chlorophylls from damage. β-carotene has also been shown to be essential for the assembly of the D1 protein into functional PSII. Given the role that carotenoids play in photoprotection by dissipating excess energy as heat through the participation of the xanthophyll cycle and the pH gradient, one would expect high levels of carotenoids in plants with a history of prolonged UV-B exposure. Contrary to this expectation however, significant reductions of carotenoids were observed in the enhanced UV-B plants. This could be an indication that the photoprotection mechanism of the plants has been compromised during prolonged exposure to UV -B radiation.

De-excitation of a molecule excited by light, occurs by fluorescence, heat, excitation energy transfer or photochemistry. At high light intensities when photosynthesis is saturated (as would be expected under conditions of high UV-B levels), unusual photochemistry can take place that can lead to damage of the photosynthetic apparatus. This could be avoided if there were a mechanism to increase energy loss as heat or fluorescence. However, during exposure of plants to high light, chlorophyll a fluorescence intensity has been shown to decrease (fluorescence quenching). One of the current suggestions is that excess light somehow promotes the formation of zeaxanthin from violaxanthin, with antheraxanthin as an intermediate. β-carotene of PSII is hydroxylated to zeaxanthin under high light stress (Trebst and Depka, 1997). It is now generally believed that it is mostly zeaxanthin (or antheraxanthin) that removes the excess energy from the excited chlorophylls and looses this energy as heat. Determination of the quantities of the pigments of the xanthophyll cycle would therefore give an indication of the plant's photoprotective capabilities.

β-carotene is essential for the assembly of the D1 protein into functional PSII. It is suspected that bleaching of β-carotene in the reaction centre of PSII by high light destabilises the structure and triggers the degradation of the D1 protein. Inhibitors of carotene biosynthesis have been found to lead to a loss of both PSII activity and D1 protein, indicating the requirement of β-carotene synthesis for the assembly of PSII in high light.

That Rubisco levels are decreased upon UV-B exposure is evident, but this has often been found to occur in the absence of any significant effects on the quantum efficiencies of PSII photochemistry. The process by which UV-B irradiation is absorbed and induces this loss of Calvin cycle enzymes is currently unknown and warrants further investigation. Several studies have demonstrated a reduction in stomatal conductance in response to UV-B irradiation. However, UV-B induced inhibition of another component of photosynthesis may result in stomatal closure in response to reduced demand for CO_2. Because of this, there is a dire need to identify direct UV-B effects on stomata of leaves relative to changes in other photosynthetic parameters. These can be done by determining the percentage decrease in light-saturated photosynthesis that is attributable to stomatal conductance (stomatal limitation). While stomatal limitation tended to be higher in UV-B irradiated leaves, the magnitude of these increases was clearly not significant to account for the large depressions in photosynthesis.

Identification of genes differentially regulated during different kinds of stresses can give clues to what defence mechanisms and biochemical pathways are regulated under these circumstances (Jordan *et al.,* 1991; Jordan, 1996; and A-H Mackerness, 2000). Methods to select for mRNAs that show differences in abundance under different conditions have been developed, such as subtractive hybridization and differential display (Liang and Pardee, 1992). With these methods, specific mRNA species, the levels of which are lowered or increased as a result of exposure of plants to UV-B radiation, can be identified. The analysis of the corresponding genes will then lead to a further understanding of UV-B effects and protective responses. However such methods suffer the drawback of giving rise to too many false positives. An alternative approach to identifying genes involved in response to UV-B is to use

RT-PCR or other techniques to clone genes homologous to those already characterised from other plant species, and investigate their expression patterns by Northern blotting. This, however, requires sequence information and function for a set of genes. Other improved techniques for finding differentially regulated genes, such as suppression subtractive hybridisation or DNA chip technology would be more effective (Brosche *et al.,* 1999; Savenstrand *et al.,* 2000). It should be noted, however, that although all the above techniques may appear to be attractive alternatives, they work best when the analysis is done concurrently with the stress factor, unlike this study in which the analysis was done in the absence of the inducing stress factor.

CHAPTER 6

Conclusions and General Discussion

The general aim of this thesis was to analyse the long-term effects of increased UV-B radiation in an indigenous plant species, and to evaluate the study's potential in providing information for the evaluation of the DNA integrity of plant seed stocks. *D. sinuata* occurs in areas where increased UV-B radiation as a result of ozone depletion, might pose a potential threat to natural plant ecosystems. Exposure to UV-B radiation has an effect both on the growing plants as well as on the dispersed seeds which lie exposed largely on barren soil surfaces for months and even years between rainfall events. It was proposed that cumulative damage to DNA might be transmitted via pollen and seeds and result in morphological, physiological and genetic changes in the plants.

Findings of UV-B induced reductions in pollen viability have been reported in several South African annual species grown under enhanced UV-B (Musil, 1995). From these findings, it has been postulated that pollen grains form an ecologically critical developmental stage of the plant, and in its natural state, *D. sinuata* pollen could lie exposed to UV-B over prolonged periods of time, and therefore is potentially vulnerable to genetic damage by UV-B. Partial purification of the enzyme photolyase from maize pollen and several types of bean, has been reported implying that repair of DNA damage to pollen is essential for survival (McLennan 1987). In the case of *D. sinuata,* if it was not repaired fully, damage to pollen may be inherited by successive generations, and thus accumulate in the genetic material.

Biochemical and physiological studies on the accumulated effects of UV-B radiation conducted on earlier generations of the study plant (Musil, 1996) had revealed some interesting observations which prompted the analyses undertaken in this study. Changes brought by accumulated UV-B included earlier reproductive effort, substantial reductions in dry mass, decreased stem and inflorescence production, diminished steady state fluorescence yields, chlorophyll-a concentrations, pollen tube

growth and germination of seed set (Musil and Wand, 1993; Musil, 1995, 1996, Midgley *et al.*, 1998). The effects of UV-B irradiation on growth and allocation of biomass appeared to be amplified as subsequent generations were exposed to UV-B irradiation pointing to the possibility that UV-B effects could be cumulative over the life history of the study plant. Furthermore, after four generations of UV-B irradiation, the effects persisted in a fifth generation that was not exposed to UV-B treatment, implying that effects of UV-B irradiation changes could be heritable. Damage to DNA caused by UV-B exposure during plant development may not be fully repaired, and thus be inherited by offspring and accumulated over successive generations in this species (Musil, 1996).

The study was aimed at establishing whether the observed accumulated effects were genetically based and also to shed some light on the long-term effects of UV-B radiation exposure in plants. To achieve this objective, three different approaches were used.

In the first approach, chloroplast DNA was studied. Green plants have an obligatory requirement for light, and as such, receive greater exposure to solar ultraviolet radiation. This light is captured by chloroplasts and by virtue of being the light harvesting machinery of the plant, chloroplasts have a significantly higher potential for acquiring ultraviolet induced genetic damage. It was therefore decided that genetic variation in chloroplast DNA between populations of plants from different latitudes would be studied. Chloroplast DNA profiles of plants that occur naturally in a high UV-B environment were compared with plants growing in an inherently lower UV-B regime. The natural population of *D. sinuata* showed evidence of changes in the chloroplast genome, but this could not be attributed to prior damage at some stage in their history by UV. This is because RFLP analysis of chloroplast DNA with restriction enzymes with different restriction sites, some being possible UV-B targets (e.g. *Dra*I) show different patterns. There is no strong evidence for UV-B, which is a form of stress, as being the cause of the observed changes. The difference in restriction pattern could not be attributed to dimer formation, but could have arisen due to evolutionary processes acting on this natural population to result in stress-induced re-arrangements of the genome. This is in agreement with findings that

chloroplast genomes usually undergo re-arrangements when subjected to stress (Manzara and Gruissem, 1988; Wakasugi *et al.,* 1997; Ries *et al.,* 2000a,b).

The second approach employed the use of the *Dra*I assay method of Harlow *et al.,* (1994) to look for direct evidence for residual dimers and residual mutations in the DNA isolated from plants derived from Generation 3 seeds grown in the absence of UV-B. This assay is based on the observation that loss of *Dra*I sites would be evidence for targeted damage by UV-B, and also indicate the presence of residual mutations in the DNA as the *Dra*I sites are likely to be hotspots for UV-B-induced mutations. Two DNA probes were used in this study, a nuclear and chloroplast probe.

The choice of a chloroplast DNA probe was because chloroplast damage would only enter subsequent generations if it occurs in the female germline. Since pollen grains have been suspected to be likely candidates for transmitting UV-B-induced DNA damage to the next generation, a nuclear *18S* rRNA probe was used. This is because although pollen may be susceptible to damage, it is only nuclear genes that would transmit nuclear damage, hence the choice of a nuclear DNA probe.

The *18S* rRNA was found to be very variable, but even though concrete deductions could not be made from these results, there is a possibility that the observed variation could have arisen from genomic rearrangements and genome instability induced by the UV-B stress. No differences were observed with the *rbc*L probe, and this agrees very well with what was seen in the natural population with chloroplast DNA analysis. This could be an indication that if the actual *rbc*L sequence is not mutated, then perhaps the regulation of photosynthetic genes has been affected, and this would explain biochemical variations observed in plant assays. To address this, the third and final approach was used which looked at differences in gene regulation in key photosynthetic pathways using mRNA studies. To understand the underlying mechanisms, a molecular knowledge of the damage is essential, so biochemical analysis of the same gene products was carried out to correlate mRNA levels with the actual gene products they encode for.

Reductions were observed in the amount of the *rbc*L mRNA expressed in the plants that have been subjected to enhanced UV-B levels. However, observed reductions did

not attain statistical significance. This could explain the reduction in net biomass and the other parameters that were observed during the physiological and biochemical analyses. The *psb*A gene on the other hand encodes the D1 protein of photosystem II, and is involved in electron transport, also linked to photosynthesis. There were also reductions in the expression of the *psb*A gene, and as was the case with the *rbc*L gene, the reduction did not attain statistical significance. Could the reduced *psb*A levels be due to a deficiency in the D1 protein itself, or does it result from some other cause, such as a regulatory mutation (resulting in the production of fewer than normal D1 molecules per cell?

There is one fascinating aspect of this study that should develop into a significant contribution to plant molecular biology in relation to UV-B effects. For a long time, it has been a well known fact that increased exposure to UV-B caused a variety of physiological and morphological responses in plants but the effect on plant genomic stability was not well established. Results from this study with plants grown in the absence of UV-B point to changes in the regulation of photosynthetic genes and such mutations due to raised UV-B levels could cause permanent changes in plant populations. These results are supported by a recent study by Ries *et al.,* (2000a) in which plants were found to be undergoing heritable and cumulative changes in the expression of genes involved in DNA metabolism.

Western blot and ELISA techniques could be used in future studies to look at how the proteins encoded by the two genes are regulated. This would give an indication of the regulation of photosynthetic genes and how they are influenced/affected by UV-B, and the level at which the regulation is being effected. Despite this plethora of information available on UV-B effects, long term effects of UV-B radiation in plants are still not well understood. Many studies need to be carried out over longer time periods to provide definitive answers to questions such as cumulative effects of UV-B, effects of UV-B at ecosystem level, and interactions of elevated UV-B with other stress factors. Plant DNA repair pathways will probably be even more important in the future if climatic predictions of ozone depletion are fulfilled. Thus an improved understanding of these pathways is important theoretically, but may also eventually have impact on global agricultural and horticultural industries.

Future studies could focus on other genes that could have a direct bearing on photosynthesis or photosynthetic pigments *e.g.* the chlorophyll a/b binding protein (*cab).* Further studies on alteration of gene expression will provide greater insight into the response of plants to UV-B radiation. For instance, additional biochemical pathways that are important for protection and stress responses may be identified. Isolation and analysis of plants unable or with diminished capabilities to synthesise either chlorophylls or xanthophylls could help explain how pigments could affect regulation of apoprotein accumulation, and the level at which this occurs (transcriptional, post-transcriptional or translational level). Analyses of the response of net CO_2 assimilation to intercellular CO_2 concentration and chlorophyll fluorescence measurements would allow evaluation of the relative limitations to leaf photosynthesis imposed by changes in stomatal conductance, carboxylating efficiency, capacity for regeneration of RuBP and PSII electron transport efficiency.

In conclusion, the results of this research indicate that there is cumulative damage at the genetic level and that this damage is heritable. It is my hope that future research based on the recommendations put forward above would shed more light on the subject. UV-B induced damage can be lethal or mutagenic and can also impede replication and transcription, a possible mechanism for the adverse effects observed in higher plants. There is no doubt that prevention of UV damage can increase plant growth and reproduction.

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